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PATHOLOGY

A Periodical Devoted to General and Experimental Pathology

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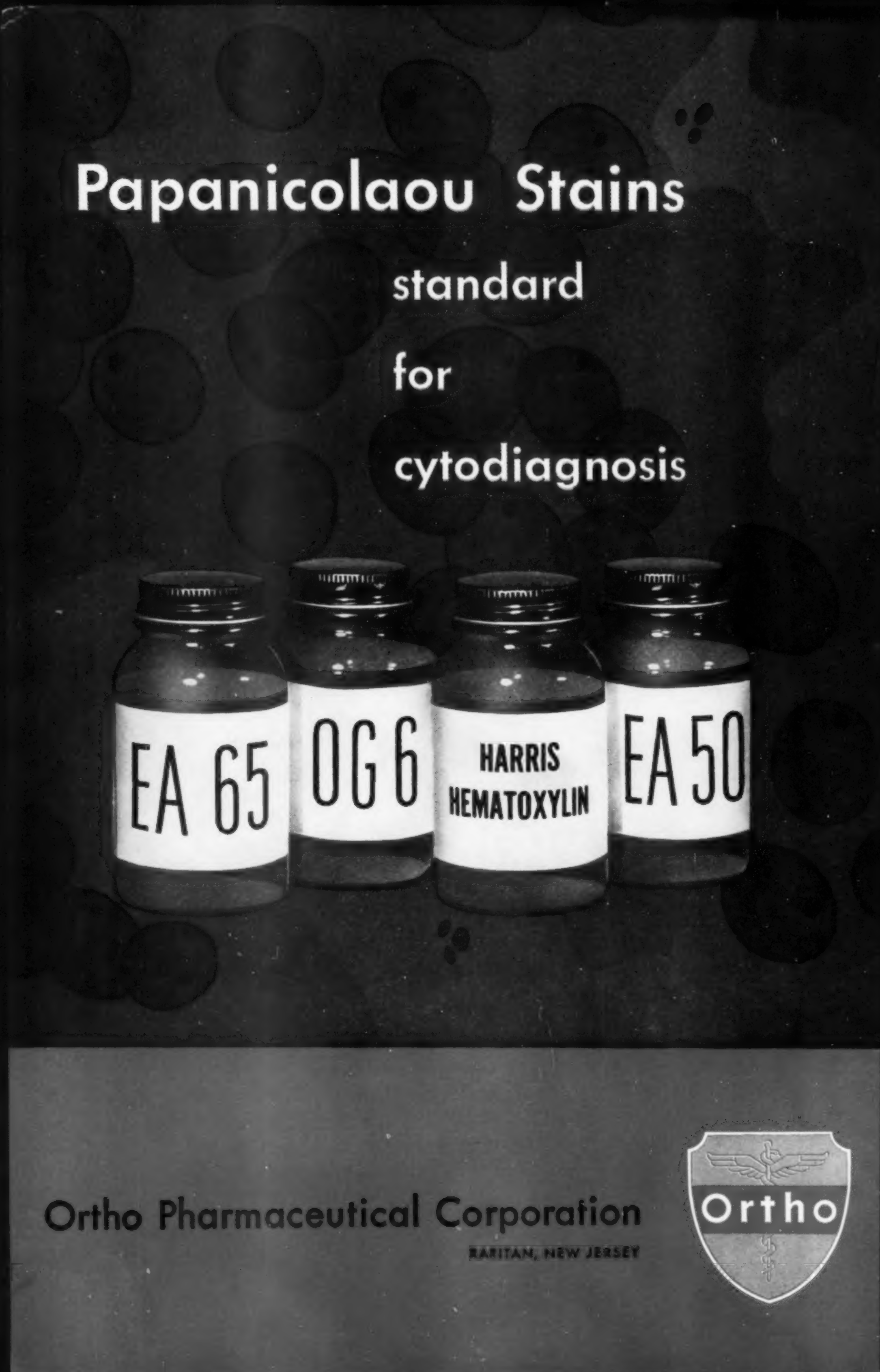
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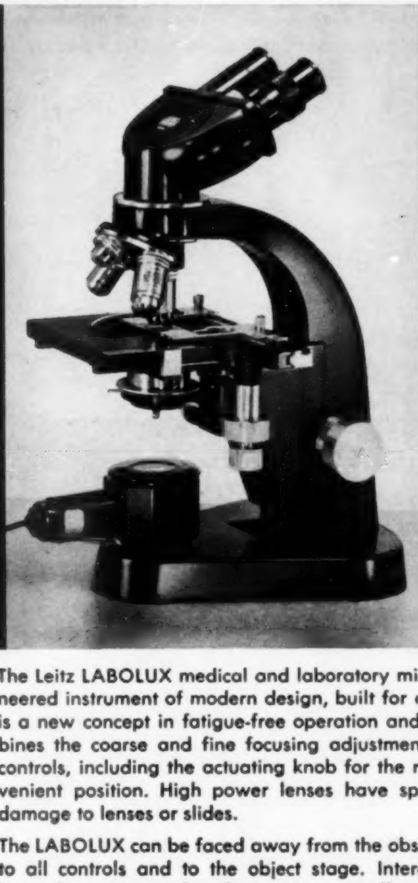

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A.M.A. ARCHIVES OF
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Mechanism of Hematopoiesis

Factors Regulating Circulatory Leukocytes

BERNHARD STEINBERG, M.D., Toledo, Ohio

Introduction

There is a fairly well-defined conviction that hematopoiesis is under the control of specific regulators. Secondary modifying factors, such as proteins, amino acids, vitamins, and arterial oxygen tension, by exerting conspicuous and recognized effects may be confused with manifestations of the regulatory mechanism.

The hematopoietic mechanism is concerned with production and maturation of blood cells, their expulsion in definite numbers into the circulation, and their disposal. One of the intriguing phases of this regulation is the maintenance of a constant number of leukocytes in the blood. The life span of mature white bloods cells in the circulation is relatively short, probably from 10 to 12 hours. These cells disintegrate in the blood as well as in certain organs, especially the spleen. The varying sites of disintegration may be of physiological significance.

As the granulocytes in the marrow and the lymphocytes in lymph nodes reach maturity, they are expelled into the circulating blood to replace those which were destroyed in the normal process of aging and death. This sequence of events suggests a quantitative relationship between cell destruction and expulsion of mature cells from the bone marrow and the lymph nodes. This study is concerned with the mechanism which controls this relationship.

In a previous investigation [1] a substance labeled "expulsion factor" was found in normal human serum and in leukocytes. This substance expelled granulocytic leukocytes from the bone marrow into the blood. The material possessed characteristics associated with an enzyme. It was postulated that this presumptive enzyme was liberated by disintegrating aging cells and acted upon the bone marrow to release a number of granulocytes proportional to the amount of enzyme released.

The expulsion factor was found to have certain physical and chemical characteristics:

> Granulocyte expulsion factor is concerned with delivery of granulocytes from marrow into blood to replace those cells which are destroyed during the process of normal aging and death. It is retained by a semipermeable membrane. It is destroyed by heating at 60 C for 30 minutes. It loses its activity when in fluid state for nine days. It is precipitated from human serum by 62% to 68% ammonium sulfate. In lyophilized state, activity is retained indefinitely. Kaolin absorption increases its activity. Concentration and purification increases its activity.

Its activity was proportional to its potency and was present in detectable amounts in blood at periodic intervals. This study of the expulsion factor was made possible by a companion one, which evaluated specific and nonspecific cellular responses to injec-

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From The Toledo Hospital Institute of Medical Research.

tion of foreign materials.[2] Several observations pertinent to this subject were obtained. Injection of foreign materials induced an early initial leukopenia which was not considered of biologic significance. It was due to vascular stasis, adhesion of cells to vascular endothelium, and redistribution in various viscera. The leukocytes became damaged, and an accelerated disintegration took place which released the "expulsion factor" and expelled cells from the marrow into the circulation, with a resultant temporary leukocytosis.

Other investigators, using different approaches, concluded directly or by implication that an "expulsion factor" is released by disintegrating cells. Doan, Zerfas, Warren, and Ames[3] injected sodium nucleinate and believed that the substance represented a stimulus which called forth granulocytes from the marrow. Weisberger, Robbins, and Heinle[4] obtained a leukocytosis-producing factor from leukocytes.

Experimental Procedures

The rabbit was the animal for all testing procedures. The serum was dialyzed. Undialyzed batches produced almost invariably circulatory collapse and inhibition of changes of the peripheral blood. The serum was injected into the marginal vein. The usual and the optimum dosage was 2.2 ml. per pound of body weight of the rabbit. In some of the experiments the dose was less, and in others the quantity was 4.4 ml. per pound. The injected material consisted of either human or bovine serum which had remained in a liquid state for not longer than seven days. The effectiveness of the expulsion factor decreased considerably after that day if the serum remained as a fluid. Much of the injected material was either in a lyophilized or a frozen state. Some animals were given injections of serum fractions obtained by precipitation with ammonium sulfate in various degrees of concentration.

The results of the effects of the injected material were determined by hourly studies of the peripheral blood over a 24-hour period. The study included the numerical counts of leukocytes, erythrocytes, platelets, and reticulocytes; hemoglobin determination by photoelectric method; microhematocrit, and differential counts of leukocytes. In some of the animals, the examinations of the peripheral blood was continued for the subsequent 48 hours at 2- to 4-hour intervals. Most of the animals were killed for histological analyses of the bone marrow and the various organs. Groups of animals were killed at various intervals following the injection of serum, and the state of the marrow and viscera was correlated with that of the peripheral blood.

Granulocyte Antiexpulsion Factor (GAEF)

An occasional animal given an injection of serum which contained tested active expulsion factor failed to respond. Many of these animals revealed a considerable hyperplasia of the lymphatic structure, especially that of the spleen. Additional observations suggested that whenever a large number of leukocytes were sequestered by the spleen, the effectiveness of the expulsion factor was reduced appreciably.

On the basis of these observations, the following hypothesis was evolved. A granulocyte antiexpulsion factor (GAEF) exists to neutralize the excessive effect of the granulocyte expulsion factor (GEF) in delivering leukocytes from the marrow into the circulation. The GAEF is elaborated by lymphatic tissue, including that of the spleen. Sequestration of leukocytes by the spleen is followed by neutralization of released GEF by concentration of locally elaborated GAEF, thus preventing expulsion of leukocytes from the marrow. Expulsion of leukocytes from the marrow initiates a series of changes in the marrow terminating in further cell production and maturation. Conversely, a lack of GEF inhibits or reduces marrow activity.

This hypothesis may be extended in principle to erythrocytes and platelets. They release their own expulsion factors which activate the marrow to expel into the circulation a corresponding number of the blood elements. The hypothesis explains some experimental and clinical problems following splenectomy in normal animals as well as in cases of leukopenia, thrombocytopenia, and pancytopenia relieved by splenectomy. The present investigations deal only with factors concerning granulocytic leukocytes.

In order to test one phase of the hypothesis, a fraction of bovine serum obtained by

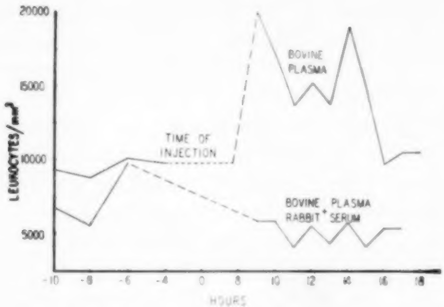

Fig. 1.—Effects of granulocyte expulsion and antiexpulsion factors in bovine serum on the peripheral blood leukocytes of rabbits. Four animals were given intravenous injections of bovine serum at zero hour, and leukocyte counts were recorded at hourly intervals. Note the rise in number of leukocytes (predominantly granulocytes) after the eighth hour. Another group of rabbits were given injections of a mixture of bovine serum (GEF) and rabbit serum (GAEF) prepared previously by injection of a serum fraction. Note the neutralization of leukocytosis by rabbit serum. Each graph is a composite of data on four rabbits.

precipitation with 0 to 34% of ammonium sulfate was injected into four animals three times over a period of three days. This fraction has been found in our investigations (unpublished)[5] to produce a lymphatic hyperplasia. The four animals were bled 12 to 18 hours after the last injection. The serum was separated and distributed in vials according to anticipated dosage. The contents of some of the vials were lyophilized.

Four rabbits were given injections of tested active bovine serum, and four other rabbits were given injections of a mixture of equal volumes of bovine serum (GEF) and lyophilized rabbit serum, presumably containing GAEF. The mixture was allowed to remain for two hours at 26 C before injection.

Four more rabbits were given injections of tested active human serum containing GEF. Another group of four rabbits received injections of equal volumes of active human serum and rabbit serum presumably containing GAEF. The mixture was left at 26 C for two hours before injection.

In these 16 animals the peripheral blood picture was determined at 2-hour intervals for 24 hours prior to injection and at hourly intervals thereafter for periods of 16 to 48 hours.

The rabbits given injections of bovine and human serum containing GEF showed variable increases in the total leukocyte content of the peripheral blood from the 8th to the 14th hour after injection. Granulocytes constituted by far the major part of the increased leukocytes.

The animals given injections of the mixtures of bovine or human serum (GEF) and rabbit serum, containing presumptive GAEF, failed to show any significant increase in peripheral leukocytes. As a matter of fact, most of the animals had a decrease of white blood cells. This decrease was present during the period in which a leukocytosis was found in the animals given injections of bovine or human serum alone containing only GEF (Figs. 1 and 2).

Injection of Rabbit Serum into Rabbits for Determination of Presence of Expulsion Factor

Injections of bovine and human serum into rabbits raised the question of the introduction of proteins foreign to the animal with its concomitant problem of the effects

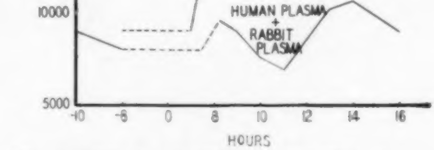

Fig. 2.—Effects of GEF and GAEF in human serum on the peripheral blood leukocytes of rabbits. Four animals were given injections at zero hour with human serum (GEF) and four other animals, with human serum and rabbit serum (GEF plus GAEF). Leukocyte counts were recorded at hourly intervals. Note the neutralization of the leukocytosis effect by rabbit serum containing granulocyte antiexpulsion factor. Each graph is a composite of data on four rabbits.

due to alien chemical substances. To evaluate the factor of a foreign protein, pooled rabbit serum was collected and injected into eight rabbits in doses of 4.4 ml. per pound of body weight.

All of the eight animals showed an increase in granulocytes approximately six to nine hours after injection. The leukocytosis lasted for an average of 10 hours. In the previous report, 10 individual samples instead of pooled serum were used.[1] Of the 10 samples, 3 were active, 5 were inactive, and 2 were doubtful.

Precipitation of rabbit serum with varying concentrations of ammonium sulfate differed in the precipitation of component proteins from that of bovine and human sera. Two fractions of rabbit serum were prepared. One fraction was the result of precipitation with 0 to 50% and the other, with 62% to 75% ammonium sulfate. The maximum effect was obtained with the 0

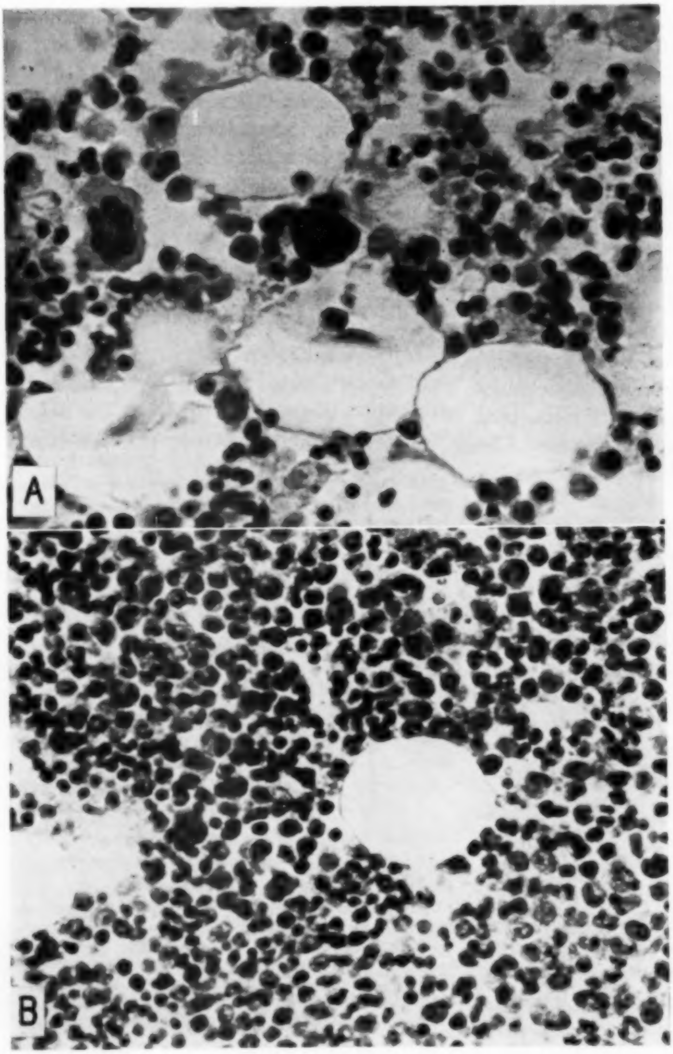

Fig. 3.—Bone marrow from humerus of rabbits after injection of GEF. *A*, four hours after injection of GEF. Note the relatively small number of cells, mostly erythroid. It is inferred that the leukocytes were expelled into the blood stream. *B*, 15 hours after injection of GEF. Note the marked hyperplasia of granulocytes which is interpreted to follow the removal of leukocytes by the action of GEF. When the effect of GEF was removed, granulocyte production and maturation was accelerated.

to 50% fraction. In the case of the human serum, the GEF was present in the 62% to 68% fraction.

Effect on Bone Marrow upon Injection of GEF

To determine the changes which may have been produced by the injection of GEF, 10 rabbits were given injections of active human serum, and the animals were killed at two-hour intervals. Paraffin sections of bone marrow of the humerus and rib, which are active, and that of the radius and ulna, normally relatively inactive, were examined. The study consisted in sample counting of 500 to 1000 cells and a general examination of the marrow sections.

In four hours, the total number of granulocytes in the marrow was decreased by 20% to 30%, owing to the reduction of mature forms. In six hours there was a marked diminution of filamented and juvenile types of granulocytes, with the total leukocyte content decreased by 40% to 55%. Marrow granulocytopenia persisted for an approximate period of 13 hours. After the 14th hour, the marrow began to show an increase in cellularity, and it reached a considerable degree of hyperplasia by the 15th hour. In the initial increase myelocytes exceeded the normal content of such cells by 30%, ±4%. The subsequent hyperplasia was contributed largely by mature forms (Fig. 3). The marrow returned to a normal state approximately 20 to 26 hours after the injection of GEF.

Examination of the bone marrow following injection of a mixture of GEF and and GAEF showed no significant changes. The study was made only on three rabbits. However, they were killed at intervals indicated by previous observations with the GEF to be illustrative of the three phases of marrow activity, that of cell reduction, hyperplasia, and a return to normal.

Effect of Adrenalectomy and Cortisone on Granulocyte Expulsion Factor

The effect of adrenals on GEF was evaluated by the injection of active human serum in three bilaterally adrenalectomized rabbits and by giving six other animals 2.5 to 4.0 mg. of cortisone daily over a period of four days prior to the injection. The basic pattern of leukocytosis in a specific number of hours did not change in either group of animals. Neither absence nor an addition of adrenal hormones affected the activity of the GEF (Fig. 4).

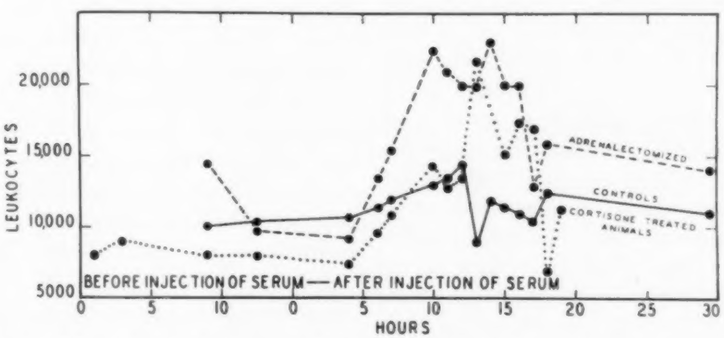

Fig. 4.—Effect of granulocyte expulsion factor on rabbits with bilateral adrenalectomy and with treatment of cortisone. The graph with straight lines is a composite of data on four normal rabbits given GEF, with a record of hourly leukocyte counts. The dotted lines represent a composite of data on three rabbits in which both adrenals were removed and injections of GEF given. The broken lines are a composite of data on six rabbits given cortisone. The basic pattern of leukocyte response was similar in the three groups of animals, indicating a lack of a significant relationship of adrenals to the GEF.

Comment

One facet of hematopoietic regulation is concerned with perpetuation of the normal number of leukocytes in the blood stream. It is generally accepted that a number of leukocytes disintegrate every few hours and new cells are delivered from the marrow to replace them. The sequence of cell destruction in the normal process of aging and replacement from depots of cell formation suggests a relationship between the two processes.

Our observations, as well as those of other investigators, suggest that the disintegrating leukocytes release a substance into the blood stream. This substance affects the bone marrow and sets up a series of events, beginning with expulsion of mature granulocytes. Subsequent changes appear to be automatic but may be determined by other regulators. The marrow shows cell depletion followed by an increased maturation and production. The final stage is a return to normal.

When several multiples of an average dose of this expulsion factor were injected into an animal, there was a corresponding increase in the degree of leukocytosis. However, upon further increase of the multiples of the unit dose, no leukocytosis took place. Apparently some other factor entered the picture to hold in check what might have developed into a leukemic or leukemoid state.

Other studies suggested the presence of another regulator, an anti-granulocyte-expulsion factor which serves to hold in check the expulsion factor. It is a matter of repeated observation that some leukocytes disintegrate in the blood and others in the spleen, the lymph nodes, the lung, and the liver.[2] It was noted in some of our work that many instances of induced leukopenia were associated with follicular and especially germ-center hyperplasia. These several observations led to the concept and the experiments which suggested that an anti-expulsion factor is elaborated by lymphatic tissue.

Summary

This study extends the concept previously advanced that the aging leukocytes upon death release a substance, probably an enzyme, labeled "granulocyte expulsion factor" (GEF). This factor acts on the bone marrow to deliver into the circulation granulocytes to replace those which were destroyed in the normal process of aging and disintegration. GEF was isolated from human serum in 62% to 68% fraction of ammonium sulfate precipitation.

The present observations suggest that GEF is not available from those granulocytes which are sequestered by the spleen and are destroyed in that organ. The studies indicate that an "anti-granulocyte-explusion factor" (GAEF) secreted by the spleen and probably other lymphatic tissue neutralizes the GEF. Some of the GAEF is delivered into the blood stream and serves to neutralize the excess of circulating GEF, thereby aiding in the maintainance of a consant number of leukocytes in the blood stream and the granulocyte-lymphocyte ratio.

These observations and concepts offer an additional explanation to the well-known occurrence of leukocytosis following splenectomy. When the remaining lymphatic tissue assumes the function of the removed spleen, the secretion of GAEF returns to normal and the leukocytes are restored numerically to a physiological state. It is not unlikely that thrombocytes and erythrocytes are controlled by a similar mechanism. The adrenals do not appear to be significantly concerned in this hematopoietic process.

The Toledo Hospital Institute of Medical Research.

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Glomus Tumor of the Uterus

ANNA M. BORGHARD-ERDLE, M.D., and EDWIN F. HIRSCH, M.D., Chicago

Glomus tumors, also described in reports as glomangiomas, angiomyoneuromas, and neuromyoarterial aneurysms, occur more often in the nail bed of digits than in the corium of the skin elsewhere. More rarely they have been reported in other structures of the body.

Glomus tumors are derived from tissues of the arteriovenous shunts or glomera of the corium. These structures have an afferent artery, the Sucquet-Hoyer canal or shunt, and an efferent vein. Several layers of small rounded glomus cells, resembling those of a nevus, surround the artery and presumably by their contractility control the flow of blood. Bundles of smooth muscle and nonmedullated nerves are in relation with the shunt.

The histologic structure of glomus tumors ranges from those composed of dense aggregates of glomus cells with only a few blood vessels to others with dilated cavernous sinuses surrounded by several layers of glomus cells. Nonmedullated nerves are usually present. Glomus tumors may have a highly organized structure.

Glomus tumors not located in the cutaneous-subcutaneous tissue junction have been reported with increasing frequency. In 1939, Iglesia de la Torre, cited by Lattes,[8] reported a painful glomus tumor in the intraphalangeal crevice of the left ring finger. Lattes and Bull [8] described a glomus tumor completely encased in the bone of the terminal phalanx of a thumb. Hoffmann and Ghormley [4] recorded a glomus tumor in the capsule of the knee joint. It was extremely painful and was excised 14 years after the onset of symptoms. German [3] described an encapsulated glomus tumor 3 by 5 cm. in the tricuspid muscle. G. V. Brindley [2] found a glomus tumor in the mediastinum of a white woman aged 29 years, associated with similar tumors in the skin of a lower extremity. The mediastinal tumor apparently was attached to the eighth intercostal nerve. Hussarek and Rieder,[5] cited by Allen,[1] described a glomus tumor of the trachea.

Kay et al.,[6] in 1951, reported three glomus tumors of the stomach. The tumors ranged in size up to 6 cm. and were located in the muscle tissues of the stomach wall. The tissue of one of these tumors was cultured, and a growth of cells resembling those that emigrate from explants of subungual glomus tumors was observed. Since then several glomus tumors of the stomach have been reported. In 1953, Spängler [12] reported 15 neurogenic tumors of the gastrointestinal tract, 1 of which was interpreted as angioneuromyoma of the stomach. The cystic tumor was 20 by 16 by 10 cm., was located along the greater curvature in the muscular layer, and was covered by serosa. The mucosal surface was ulcerated. Allen and Dahlin,[1] in a review of the records concerning benign tumors of the stomach in the files of the Mayo Clinic, found two glomus tumors, both in Negroes. Mannix et al.[9] added another glomus tumor of the stomach. The first glomus tumor of the stomach reported from France was recorded in February, 1956, by J. Lamy and H. Bonneau.[8] It was the size of a walnut and grossly was cystic in the center and fibrous at the periphery. About the same time Rubens-Duval et al.,[11] in France, published another report of a glomus tumor of the stomach, 4 cm. in diameter.

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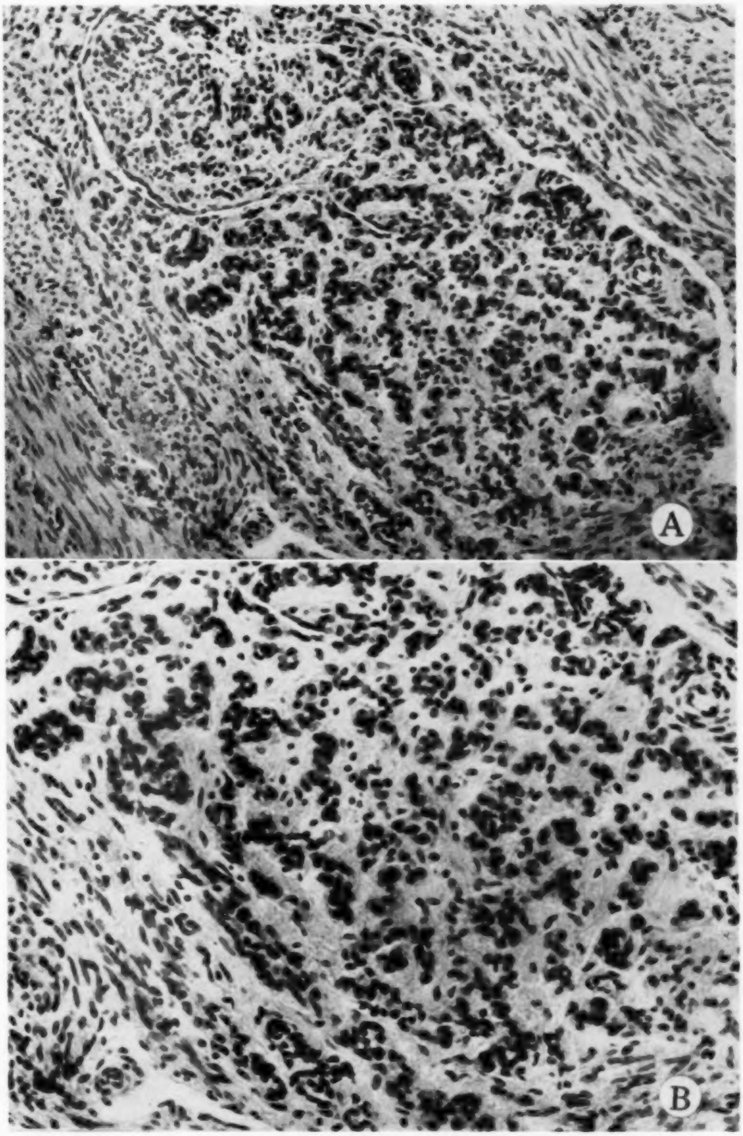
From the Henry Baird Favill Laboratory of St. Luke's Hospital.



Photomicrograph illustrating *A*, the entire mass of the glomus tumor in a section and *B*, a higher magnification of the midportion of the tumor, to give more detail and arrangement of the small cells. Note the capillaries at the left upper edge of each illustration, the muscular artery at the right upper edge of the tumor, the groups of small glomus cells, and a segment of nerve fiber at the lower edge of the tumor.

We have not found a published report of a glomus tumor of the uterus.

The clinical symptoms of the tumors vary with the size and location. All are described as benign. Histologically they have the characteristic organoid appearance of a glomus with small or dilated capillaries surrounded by the typical round glomus cells and associated with nonmedullated nerve fibers.

Report of a Case

In the routine examination of surgical tissues a small glomus tumor was found in the wall of a uterus removed from a white woman aged 49 years, a secundipara, who entered St. Luke's Hospital for increased menstrual flow of two months' duration without pain. The uterus was enlarged, and a vaginal hysterectomy was done. The uterus had several fibromyomas, ranging up to 5.5 cm. in maximum diameter.

The microscopic examination of sections from one block disclosed a small circumscribed but not encapsulated cellular tissue mass in the myometrium 4 mm. beneath the proliferative endometrium. This block was cut serially. The nodule was only 3 to 4 mm. in diameter and consisted of densely packed cell clusters associated with compressed blood capillaries (Figure). The cells were small, round, occasionally elongated, and well defined. They had a large round vesicular nucleus with a distinct chromatin structure. The cell clusters surrounded capillaries and were in spaces between the capillaries. Some cell masses did not have a central blood vessel. In sections stained with Foot's silver stain the capillary walls were clearly defined and the previously mentioned cell clusters were outside of the capillary walls.

The reason for the occurrence of glomus tumors at sites where normal glomera are not found regularly may be that glomera are distributed more widely than has been supposed but so sparsely as to escape detection. Murray and Stout,[10] in an excellent study, offered an explanation for the occurrence of glomus tumors in uncommon sites. The epithelioid cells of the glomus tumor are regarded generally as modified smooth-muscle cells which are able to constrict the glomus vessel. Through tissue cultures Murray and Stout identified the epithelioid cell of the glomus tumor with the capillary pericyte of Zimmermann [13] (cited by Murray and Stout [10]), which is normally present in many parts of the body. These pericytes, described in 1923 by Zimmermann,[13] are distinct from adventitial cells and are contractile.

The glomus tumors are closely related to the hemangiopericytoma. They differ only slightly in histologic structure. The glomus tumor has round epithelioid cells filling the space between capillaries. These occasionally are interrupted by a loose stroma packed with nonmedullated nerve fibers. Hemangiopericytomas in addition to many obvious capillaries have a profuse proliferation of occult capillaries.

Summary

A small glomus tumor of the myometrium of the uterus is described.

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Experimental Arthritis

III. Modification of Acute Lesions in the Guinea Pig by Corticotropin (ACTH) and Steroids

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Acute arthritic lesions are induced in guinea pigs by certain bacterial polysaccharide complexes injected by subcutaneous,[1] intravenous,[1,2] or intraperitoneal [2] routes. The joint lesions are characterized by an initial increase in metachromatic mucoid material followed by increasing quantities of nonmetachromatic cell-free exudate and by synovial proliferation. C^{14}-labeled polysaccharide complexes from Klebsiella pneumoniae, Type B, injected in milligram quantities, reach the joint fluid and remain within synovial cells for some weeks.[3] Since corticotropin (ACTH), cortisone, hydrocortisone (Cortisol) and related steroids have been employed in the treatment of such diseases as rheumatoid arthritis and rheumatic fever, the present study on steroid modification of the experimental arthritic lesions in the guinea pig was undertaken.

Materials and Methods

Animals.—Guinea pigs of either sex, weighing 180 to 250 gm. at the initiation of the experiment and free of streptococcal lymphadenitis, were fed Purina Rabbit Chow with daily supplements of cabbage. The animals were weighed twice per week.

Bacterial Polysaccharide.—The polysaccharide complex used in these experiments was obtained by extraction of K. pneumoniae, Type B, with 0.01 N KOH.[1,2,4] This polysaccharide is a nonlethal hapten which does not produce bile precipitate, hepatic basophilia, or ascites but which does produce joint lesions.[3] The dry polysaccharide was heat-sterilized and then prepared as a 1% solution in sterile 0.85% NaCl. The guinea pigs were given a single injection of 1 mg. of the polysaccharide per 100 gm. of body weight and killed either two or seven days later.

Steroids.—Most groups of guinea pigs were pretreated with hormones for seven days prior to the polysaccharide and for the subsequent period of two or seven days. Guinea pigs killed seven days after the injection of polysaccharide were given injections subcutaneously each day with 3 mg. per 100 gm. of body weight of the following substances: Δ^5-pregnen-3-β-ol-20-one (pregnenolone) in sesame oil; progesterone in sesame oil; 11-desoxy-17-hydroxycorticosterone (Reichstein's Compound S) in sesame oil; desoxycorticosterone in sesame oil; corticosterone (Kendall's Compound B) in Merck vehicle No. 1. Animals in other groups were given cortisone, 1 and 3 mg. per 100 gm. of body weight, in Merck vehicle No. 1; cortisone, 3 mg. per 100 gm., plus corticotropin, 3 I. U. per 100 gm. in 1% gelatin, and corticotropin, 3 I. U. per 100 gm. of body weight. All groups of guinea pigs which were killed two days after a single intravenous injection of polysaccharide were pretreated with seven daily subcutaneous injections of 3 mg. or 3 I. U. per 100 gm. of body weight of corticosterone in Merck vehicle No. 1, cortisone in Merck vehicle No. 1, corticotropin in 1% gelatin, or cortisone plus corticotropin and desoxycorticosterone acetate (DOCA) in Merck vehicle No. 1. Additional groups of guinea pigs were given daily injections of cortisone, 1 mg. per 100 gm., and corticotropin, 3 I. U. per 100 gm., beginning at the time of the single injection of polysaccharide, and killed at seven days.

Controls.—Guinea pigs receiving no injections were killed at various times during the experimental study. Another group received daily subcutaneous injections of 0.85% NaCl. Other controls received only polysaccharide, vehicle No 1, sesame oil, corticosterone, desoxycorticosterone, cortisone, or corticotropin. Daily injection of vehicle No. 1 was given one week before and one

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week after the single intravenous injection of bacterial polysaccharide.

Morphologic Studies.—All tissues were fixed in 10% formalin in acetone. Both elbows, a shoulder, one or both knee joints, and a costochondral juncture were examined in all animals. After decalcification with 5% HNO_3, paraffin sections were prepared and stained with hematoxylin and eosin. Duplicate sections were stained with toluidine blue, Hale's colloidal iron plus periodic acid leukofuchsin (PAS),[6] and alcian blue plus PAS. The histologic changes were evaluated without knowledge of the experimental conditions to which the animal had been subjected. An accurate quantitation of the joint changes was not possible; the arbitrary grading of severity of a specific histologic change ranged from slight to 4+. The types of recorded histologic changes were (*a*) metachromatic mucoid substance (homogeneous, reticular or dense), (*b*) nonmetachromatic eosinophilic material in reticular or granular form, (*c*) similar material in larger globular or irregular masses, (*d*) dense eosinophilic exudate with fibroblasts, (*e*) synovial proliferation with radicular or nonradicular structures or at unusual sites.

At autopsy careful observation was made for retroperitoneal edema, ascites, hemorrhages in adrenals, necroses in abdominal fat,[8] distention of the gallbladder, bile precipitate, and size of the cervical and mesenteric lymph nodes and thymus. Brain, cervical and mesenteric lymph nodes, thymus, salivary gland, lung, heart, liver, gallbladder, spleen, adrenal, kidney, and ovary or testis were examined histologically.

Results

Joint Changes with Bacterial Polysaccharide.—The changes which are seen within the joints in guinea pigs after a single intravenous injection of Klebsiella polysaccharide complex have been described in two previous studies.[1,2] Modifications in the joint lesions encountered in the present study with the steroids necessitate amplification of the description of these changes.

Three types of noncellular material which appear within the joint space are readily distinguished by morphologic and staining characteristics (Table 1). The basophilic material is retained in the joint space with acetone-formalin fixation. This basophilic material is metachromatic with toluidine blue and deep blue with the Hale-collodial iron or with alcian blue. After acetone-formalin fixation and paraffin embedding, this material is usually filamentous or reticular, but may appear homogeneous (Fig. 1) or as a very dense mass (Fig. 2). With toluidine blue, the fine strands are metachromatic, but occasionally metachromatic granules are noted along the delicate orthochromatic or almost unstained strands. Minute PAS-positive granules often appear along the metachromatic strands. The uniform staining of the strands with colloidal iron or alcian blue and the metachromasia disappear after testicular hyaluronidase in phosphate buffer at pH 7.0 for 12 hours at 37 C. This metachromatic material is tentatively identified as hyaluronic acid, the only polysaccharide found in synovial fluid.[7]

The eosinophilic, PAS-positive material has several forms: (*a*) a fine granular to fibrillar material interspersed among the metachromatic material (Fig. 7); (*b*) globules of varying size (Fig. 3), often studded with metachromatic granules (Fig. 4); (*c*) larger irregular masses free in joint space, attached to the synovial surface or covering the articulating cartilage (Fig. 10). The staining properties of this material remain unchanged after treatment with hyaluronidase.

The third type of material does not stain metachromatically or with PAS, colloidal iron, or alcian blue but does stain blue with phosphotungstic acid-hematoxylin. Apparently this material is fibrin (Fig. 6). This exudate is often seen as a loose meshwork

TABLE 1.—*Staining Characteristics*

Hematoxylin and Eosin	Toluidine Blue	Colloidal Iron or Aician Blue	Periodic Acid-Leucofuchsin (PAS)	Testicular Hyaluronidase
A. Basophilic, reticular-granular	Metachromatic	Positive	Negative	Disappears
B. Eosinophilic, reticular-granular C. Globules and masses	Orthochromatic (variable intensity; occasional slightly metachromatic)	Negative	Positive (intense)	No change
D. Dense eosinophilic exudate with scattered fibroblasts	Orthochromatic (pale)	Negative	Negative	No change

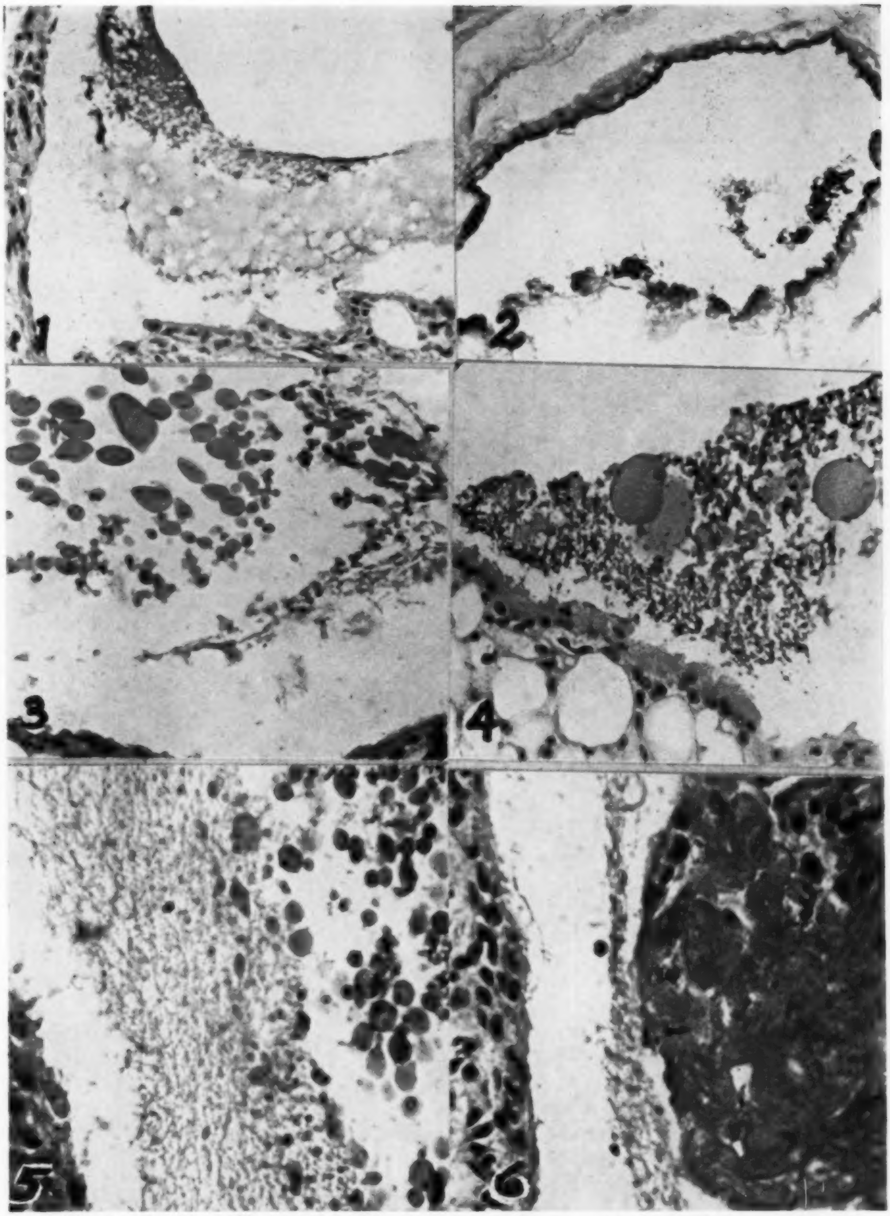

Fig. 1.—The basophilic material often appears as a homogeneous mass (lower area) or as granular and filamentous material (upper area); scapulohumeral area in guinea pig given corticotropin one week before and one week after a single intravenous injection of K. pneumoniae polysaccharide complex. Hematoxylin and eosin; × 200.

Fig. 2.—Scapulohumeral (shoulder) joint stained to show the metachromasia of the synovial cells and fluid. Toluidine blue; × 110.

Fig. 3.—Eosinophilic spheroidal masses are PAS-reactive and orthochromatic and are not decreased in the joint by cortisone one week before and two days after a single intravenous injection of polysaccharide. Hematoxylin and eosin; × 250.

Fig. 4.—Granular-reticular metachromatic material, apparently hyaluronic acid or a complex of it with protein, is increased by one week before and one week after treatment; the metachromatic granules stud the orthochromatic spheroidal masses. Hematoxylin and eosin; × 250.

Fig. 5.—The humeroulnoradial (elbow) joint shows intermingling of eosinophilic globules and the granular-reticular strands in a guinea pig receiving only polysaccharide; proliferated synovial cells are seen in the lower left. Hematoxylin and eosin; × 300.

Fig. 6.—Dense eosinophilic orthochromatic, PAS-negative material, apparently fibrin, interspersed with fibroblastic cells; to the left of the exudate are strands of metachromatic material and, beyond this, the synovial cells. Hematoxylin and eosin; × 300.

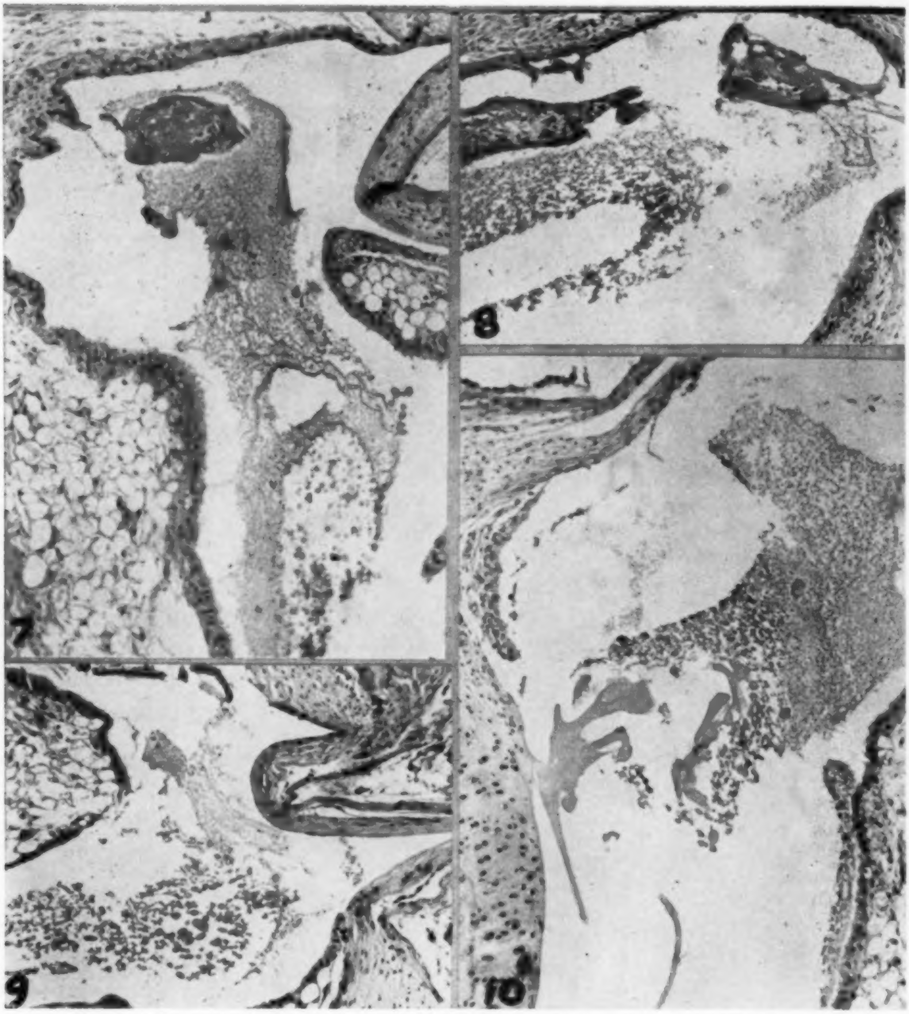

Fig. 7.—The various changes in the elbow joint after intravenous polysaccharide are (*a*) the fibrinous exudate (upper area); (*b*) basophilic metachromatic material, apparently hyaluronic acid; (*c*) the eosinophilic, PAS-reactive globules (lower area), and (*d*) the proliferation of synovial cells. Hematoxylin and eosin; × 75.

Fig. 8.—Desoxycorticosterone acetate given subcutaneously, 3 mg/100 gm. body weight per day, one week before and one week after the bacterial polysaccharide, shows less basophilic metachromatic material but no significant change in the amount of eosinophilic globules; fibrinous material is seen in upper right. Hematoxylin and eosin; × 75.

Fig. 9.—Cortisone one week before and two days after the polysaccharide may be associated with focal (upper left) but not a general increase in synovium. Some metachromatic material lies near the eosinophilic globules (low-power magnification of Fig. 3). Hematoxylin and eosin; × 75.

Fig. 10.—Corticotropin one week before and one week after the bacterial polysaccharide shows a large amount of metachromatic material without reduction in the eosinophilic masses and globules. Hematoxylin and eosin; × 75.

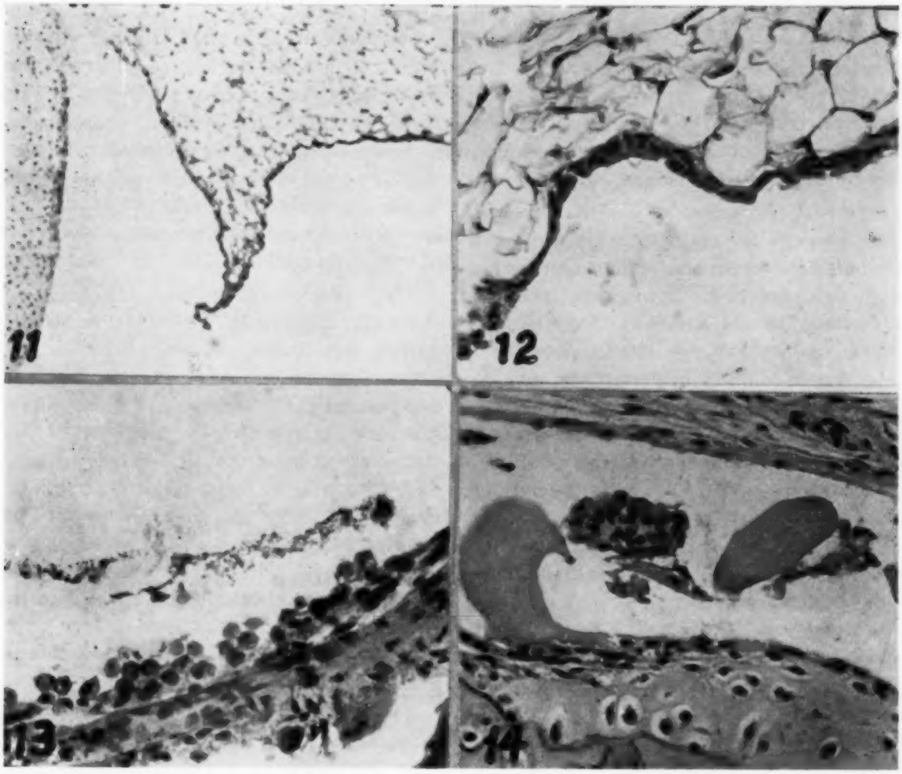

Fig. 11.—The synovial cells form a thin layer over underlying adipose cells in the knee joint of a guinea pig not given an injection. Hematoxylin and eosin; × 75.

Fig. 12.—Higher magnification of Figure 11. Hematoxylin and eosin; × 250.

Fig. 13.—Synovial cells are vacuolated, rounded, and apparently detaching in a cortisone-pretreated guinea pig given one injection of bacterial polysaccharide. Hematoxylin and eosin; × 250.

Fig. 14.—Eosinophilic masses lie free in the joint space and over the articulating surface of cartilage; the central group of synovial cells represent a transected papillary projection. Hematoxylin and eosin; × 250.

at two days following the injection of polysaccharide, but at seven days the material is more compact and interspered with fibroblastic cells. A frequent site of attachment of this exudate is at the extreme reflection of the joint space (Fig. 7, 8).

Vacuolization of the synovial cells and proliferation of synovial cells have been described previously.[2] For simplification of tabulation in the present report, all proliferative changes of synovium are grouped together. These proliferative changes include a general increase in cells, a focal polypoid hyperplasia, or elongated, pannus-like extensions. The granules of the synovial cells (Fig. 13) are not prominent and are usually very faintly metachromatic in the center of a colorless refractile zone. Such granules are generally the most numerous near the surface of the synovium.

The most marked changes of all types usually are found in the humeroradioulnar (elbow) joints, with mildest change in the scapulohumeral (shoulder) joints and changes of intermediate degree in the femorotibial (knee) joints.

Control animals not given injections have very scanty basophilic, metachromatic, and

orthochromatic material in the joint space. In control, as well as in experimental, animals, the basophilic material is more frequently encountered in the shoulder joint. The synovial cells of the shoulder joints are less prominent but more often metachromatic than the cells of the knees or elbows.

Effect of Hormones on Joint Changes.—The effects of the corticosteroids and corticotropin pretreatment upon the joint lesions of guinea pigs given a single injection of polysaccharide are given in Table 2 and summarized in Table 3. The incidence of a joint change is given in percentage, and the degree is graded from slight (+) to severe (4+). Joint changes in animals given only the polysaccharide are considered as mild to moderate (2+ to 3+). The untreated and the control animals given injections of saline have slight (+) metachromatic material and no or slight eosinophilic granules and strands without globules or masses. Sesame oil but not Merck vehicle controls show a slight amount of the eosinophilic globules in the elbow joint. Animals given only the corticosterone also show this response and have mild amounts of fibrinous exudate as well. Occasionally a slight amount of eosinophilic globular material is found in the cortisone, corticotropin, and desoxycorticosterone acetate controls.

Two days after the polysaccharide injection, the metachromatic material, apparently hyaluronic acid, is decreased in incidence and quantity in the cortisone and/or corticotropin groups and mildly decreased in amount in the desoxycorticosterone acetate pretreated group. At seven days there is a striking increase in the metachromatic material in the cortisone and corticotropin groups, but this is not seen in the cortisone plus corticotropin group. The metachromatic material in corticosterone and pregnenolone groups is similar to that of the animals given only the polysaccharide, but in the progesterone and Compound S groups the material appears diminished.

The eosinophilic, PAS-positive material, especially the globules and masses, is augmented at two days in the corticosterone group, unchanged in the cortisone and corticotropin groups, but decreased in the cortisone plus corticotropin pretreated guinea pigs. At seven days both the cortisone plus corticotropin and the corticotropin group are similar to the controls given polysaccharide injections, while the corticosterone and desoxycorticosterone acetate groups show less of the eosinophilic globules and masses.

The nonmetachromatic, PAS-negative material, apparently fibrin, is found in guinea pigs given the polysaccharide and pretreated with corticotropin, corticosterone, and cortisone. The incidence of synovial proliferation is not greatly modified by the steroids or corticotropin. However, cortisone and, to a smaller extent, corticotropin decreases the over-all prominence of the synovial cells, while the focal areas of proliferation are still evident; therefore, the incidence of proliferation (Table 2) in the relatively atrophic synovium is similar to that of the controls given polysaccharide injections.

The order of change in the different joints in animals given steroids or corticotropin plus polysaccharide is the same as with the polysaccharide only, the elbow showing the greatest and the shoulder, the least alteration.

The increase in metachromatic hyaluronic acid with corticotropin and cortisone is not dependent upon pretreatment, for the same effect is attained when these hormones are begun at the time of a single injection of bacterial polysaccharide complex (Table 2). There may be a mild decrease in synovial cells with cortisone and cortisone plus corticotropin controls, but even in these animals the addition of the polysaccharide induces focal proliferation and vacuolization of synovial cells (Fig. 13).

Changes in Other Tissues.—Corticotropin plus cortisone for 10 days leads to the accumulation of protein-rich ascitic fluid.[6] When the single intravenous injection of polysaccharide is given several days before corticotropin- and cortisone-treated animals

Table 2.—*Effects of Various Steroids and Corticotropin Upon Joint Lesions Induced by a Single Intravenous Injection of K. Pneumoniae Polysaccharide Complex**

	Substance Day/100 Gm. Body Weight (Subcutaneously)	Days Pretreatment	Day Killed After Polysaccharide	No. Animals	Elbow: No. Joints	A † %	A † Degree	B ‡ %	B ‡ Degree	C § %	C § Degree	D ‖ %	D ‖ Degree	Synovial Proliferation %	Synovial Proliferation Degree
Polysaccharide control	0	--	2	6	12	67	2-3+	67	2-3+	67	2-3+			50	2-3+
Polysaccharide control	0	--	7	7	14	93	+–4+	78	+–4+	57	2-3+			71	2-3+
Sesame oil only	0.3 ml.	--	--	4	8	71	+	57	+	43	+			63	+
Merck vehicle & polysaccharide	0.3 ml.	7	7	7	11	100	+–4+	64	2-3+	64	2-3+			36	2-3+
Cortisone only	3 mg.	--	--	5	10	70	+	50	+	40	+			30	+
Cortisone & polysaccharide	3 mg.	7	2	5	10	30	+	70	+	60	+–4+	10	+	50	+
Cortisone & polysaccharide	3 mg.	7	7	8	14	71	4+	43	2-3+	64	+–4+			43	+
Cortisone & polysaccharide	1 mg.	7	7	6	11	82	2-3+	73	+–4+	73	+–4+			91	+
Cortisone & polysaccharide	3 mg.	0	7	4	8	100	2-3+	75	+–4+	38	+–3+	25	+	25	+
Corticotropin only	3 I.U.	--	--	5	10	50	+	60	+	30				50	+
Corticotropin & polysaccharide	3 I.U.	7	2	6	11	100	+	55	+	73	+–4+	36	+–3+	82	+–3+
Corticotropin & polysaccharide	3 I.U.	7	7	10	19	95	4+	63	2-3+	84	2-3+	16	2+	63	+–3+
Corticotropin & polysaccharide	3 I.U.	0	7	7	14	100	4+	79	2-3+	64	2-4+	7	2+	64	+–3+
Cortisone & corticotropin only	3 mg. & 3 I.U.	--	--	5	10	50	+	70	+	10	+			20	+
Cortisone, Corticotropin & polysaccharide	3 mg. & 3 I.U.	7	2	5	9	67	+	67	+	56	+				
Cortisone, Corticotropin & polysaccharide	3 mg. & 3 I.U.	7	7	5	9	70	2-3+	78	+–4+	78	2-3+	22	2-3+	22	+
Corticosterone only	3 mg.	--	--	5	10	20	+	90	2-3+	50	+	60	+		
Corticosterone & polysaccharide	3 mg.	7	2	12	24	83	+–4+	100	+–4+	63	4+	21	2-3+	63	2-3+
Corticosterone & polysaccharide	3 mg.	7	7	13	26	77	+	88	+	38	2-3+			62	2-3+
Desoxycorticosterone acetate only	3 mg.	--	--	5	10	30	2-3+	90	+–4+	50	+			45	+
Desoxycorticosterone acetate & polysaccharide	3 mg.	7	2	5	10	90	+	70	+–4+	60	2-3+			60	+
Desoxycorticosterone acetate & polysaccharide	3 mg.	7	7	7	14	57	2-3+	29	2-3+					100	2-3+
Compound S & polysaccharide	3 mg.	7	7	5	10	30	+–3+	50	+–3+	40	+–2+			80	2-3+
Pregnenolone & polysaccharide	3 mg.	7	7	5	10	80	+–4+	75	+	30	+–3+			80	2-3+
Progesterone & polysaccharide	3 mg.	7	7	5	9	56	+	56	3-4+	45	+–3+			56	

* 1 mg./100 mg. body weight.
† Metachromatic, alcian blue-positive, usually basophilic strands (hyaluronic acid?).
‡ Eosinophilic orthochromatic, PAS-reactive strands and fine granules.
§ Eosinophilic, PAS-reactive globules and masses.
‖ Orthochromatic, PAS-negative exudate (fibrin?).

TABLE 2.—*Effects of Various Steroids and Corticotropin Upon Joint Lesions Induced by a Single Intravenous Injection of K. Pneumoniae Polysaccharide Complex** Continued

	Knee										
	No. Joints	A †		B ‡		C §		D ‖		Synovial Proliferation	
		%	Degree	%	Degree	%	Degree	%	Degree	%	Degree
Polysaccharide control	8	50	2-3+	50	+-4+	50	+-4+			75	+-4+
Polysaccharide control	6	50	2-3+	83	2-3+	50	2-3+			50	2-3+
Sesame oil only											
Merck vehicle & polysaccharide	6	50	2-3+	50	2-3+	33	2-3+			33	2-3+
Cortisone only	10	50	+			20	+	10	+	20	+
Cortisone & polysaccharide											
Cortisone & polysaccharide	9	56	+-4+			22	2-3+			22	+
Cortisone & polysaccharide	6	67	2-3+	33	2-3+					50	+
Cortisone & polysaccharide	4	75	2-3+	25	2-3+	25	2-3+			25	2+
Corticotropin only	5	20	2-3+	40	2-3+	20	+				
Corticotropin & polysaccharide	6	84	+-4+	67	2-3+	50	+	50	+	33	2+
Corticotropin & polysaccharide	10	90	4+	30	2-3+	50	2-3+			10	2+
Corticotropin & polysaccharide	7	100	4+	23	2-3+	23	2-3+			57	2-3+
Cortisone & corticotropin only	10	10	+	20	+	19	+				
Cortisone, Corticotropin & polysaccharide	10	50	2-3+	50	2-3+	20	+			20	+
Cortisone, Corticotropin & polysaccharide	7	57	2-3+	29	2-3+	14	2-3+	14	+		
Corticosterone only	9	33	2-3+	67	2-3+	56	+	11	+		
Corticosterone & polysaccharide	4	100	+	75	2-3+	50	2-3+			75	2-3+
Corticosterone & polysaccharide	9	100	+	89	2-3+	22	2-3+			45	2-3+
Desoxycorticosterone acetate only	10	60	2-3+	50	2-3+	30	+	10	+	30	+
Desoxycorticosterone acetate & polysaccharide	6	33	2-3+	50	2-3+	33	2-3+			33	2-3+
Desoxycorticosterone acetate & polysaccharide	4			75	2-3+	25	2-3+			75	2-3+
Compound S & polysaccharide	5	20	2+	40	2+	20	2+			80	2-3+
Pregnenolone & polysaccharide	5	30	+-3+	40	2-3+					40	2-3+
Progesterone & polysacchar'de	5	20	2-3+	40	2-4+	20	2-3+			40	2-3+

	Shoulder										
	No. Joints	A †		B ‡		C §		D ‖		Synovial Proliferation	
		%	Degree	%	Degree	%	Degree	%	Degree	%	Degree
Polysaccharide control	6	67	+	33	+	33	+				
Polysaccharide control	5	40	2-3+	60	+			20	2+	20	2+
Sesame oil only	4	50	+	50	+						
Merck vehicle & polysaccharide	9	78	2-3+	56	2-3+			11	2+	22	2+
Cortisone only	5	20	+	60	+-4+						
Cortisone & polysaccharide											
Cortisone & polysaccharide	6	67	2-3+	33	2-3+	17	2+				
Cortisone & polysaccharide	8	88	2-3+			38	2+			38	+
Cortisone & polysaccharide	4	100	2-3+			25	2+				
Corticotropin only	7	52	+	52	+						
Corticotropin & polysaccharide	12	92	+-4+	33	2-3+	33	2-3+			25	+
Corticotropin & polysaccharide	10	80	+-4+	10	+	10	2+			30	+
Corticotropin & polysaccharide	7	86	4+								
Cortisone & corticotropin only	5	20	+	20	+						
Cortisone, Corticotropin & polysaccharide	6	50	+	50	2-3+						
Cortisone, Corticotropin & polysaccharide	7	14	2-3+	14	2+	14	2+				
Corticosterone only	5	20	2+	60	2-3+	20	2+				
Corticosterone & polysaccharide	10	40	2-3+	70	2-3+	20	2-3+			50	+
Corticosterone & polysaccharide	13	77	2-3+	77	2-3+						
Desoxycorticosterone acetate only											
Desoxycorticosterone acetate & polysaccharide											
Desoxycorticosterone acetate & polysaccharide											
Compound S & polysaccharide	5	40	2-3+	40	2-3+						
Pregnenolone & polysaccharide	5	50	2-3+	20	2+					20	2+
Progesterone & polysaccharide	6	17	2+	50	2-3+	17	2+			33	2+

TABLE 3.—*Summary of Effects of Steroids and Corticotropin Upon Acute Joint Lesions in Guinea Pigs Given Polysaccharide* *

	2 Days				7 Days			
	A	B	C	D	A	B	C	D
Cortisone	↓	→↓	→	+	↑	→↓	→	0
Corticotropin	→↓	↓	→	+	↑	→	→	+
Cortisone & corticotropin	→↓	→↓	↓	0	→	→	→	+
Corticosterone	→	↑	↑	+	→	↓	→↓	0
Desoxycortico-sterone acetate	→↓	→	→	0	↓	↓	↓	0

* → same as in animals given polysaccharide injections; →↓ same as polysaccharide controls in one joint, decreased in another; + lesion present (no lesion in polysaccharide controls); 0 lesion absent

are killed, the accumulation of ascitic fluid is more marked. Corticosterone alone leads only to the minimal joint changes indicated above, but when the bacterial polysaccharide is given to the corticosterone-pretreated animal, "toxic" responses occur. These toxic responses include the distention of the gallbladder, the precipitate of bile, ascites, retroperitoneal exudate, adrenal hemorrhages, intussusception of the distal colon, and death.[2] Death and intussusception occur only during the first 48 hours after the polysaccharide, but the other changes may be seen 7 days later. The haptenic, polysaccharide complex used in the present experiment rarely leads to deeply basophilic particles in the liver cord cells.[2] Animals receiving corticotropin, but not corticotropin plus cortisone or the other steroids, had varying amounts of these particles. Large pale vacuolated liver cells, containing abundant PAS-positive diastase-removable glycogen, were observed in the cortisone and corticotropin plus cortisone groups, and these liver cells often had areas of very pale basophilia. The histologic changes in adrenals, lymph nodes, spleen, etc. are similar to those already described.[2]

Comment

The C^{14} from intravenously injected labeled bacterial polysaccharide reaches the joint fluid and localizes in the synovium in high concentration.[3] In autoradiographs the concentration in the synovium appears much greater than in the bone marrow of the same section.[3] This suggests that the changes in joint fluid and the proliferation of synovial cells may be the direct effect of localization of foreign polysaccharide in synovial cells or fluid. Because it has not been possible to quantitate accurately the C^{14} in the synovium or joint fluid, the effects of steroids and corticotropin upon the rate of appearance and disappearance of the labeled polysaccharides in the synovial structures remains unknown.

The present study indicates that corticotropin and cortisone alone or in combination lead to a delayed increase in metachromatic material, apparently hyaluronic acid. Eosinophilic, PAS-reactive material is augmented by corticosterone and is increased or unchanged but not reduced by the other steroids or corticotropin.

Hyaluronic acid[7] and proteins comprise the macromolecular components of the synovial fluid of man, cow, and horse. The proteins are apparently derived from plasma in that they have the same relative proportions,[8-10] electrophoretic mobilities,[11,12] and immunologic properties[12] as plasma proteins. However, since the origin of the plasma protein is obscure, some of the protein could originate from the synovium. Hyaluronic acid, the only mucopolysaccharide identified in synovial fluid, is apparently derived from synovial cells, and consists of the disaccharide, N-acetylhyalobiuronic acid,[13] polymerized into unbranched, flexible, chain molecules which coil to form highly hydrated spheres of large diameter.[14] The high negative charge of the hyaluronic acid explains its affinity for water and cationic proteins.[14]

The synovial space has been compared to a connective tissue space filled with its liquid matrix.[15,16] The present discussion cannot encompass the extensive literature on the biologic and chemical effects of steroid hormones upon connective tissue, but two aspects common to synovium and connective tissues—the mucopolysaccharides and their cells of origin—are pertinent. Unlike the

synovial fluid with only one mucopolysaccharide, hyaluronic acid, the subcutaneous tissue and dermis have several—hyaluronic acid, chondroitin sulfuric acid, and uronic acid-free polysaccharides comprised of galactose, mannose and fucose.[17,18] The isotopes S^{35} and C^{14} have been employed in the attempt to study the metabolism of chondrotin sulfate and hyaluronic acid of skin. Since $S^{35}O_4$ is fixed as the ester sulfate in chondroitin sulfate[19] and C^{14}-carboxyl-labeled acetate is incorporated as the N-acetyl group of hyaluronic acid and chondroitin sulfate,[20] neither isotopic study may indicate the actual rate of synthesis of the molecular "skeleton." Schiller et al.[20] using C^{14} in N-acetyl group as an index, found that hyaluronic acid in rabbit skin has a half-life of about two days and is formed three times more rapidly than chondroitin sulfate. Layton[21] found that cortisone reduced the incorporation of S^{35} in the mucopolysaccharides of the skin of the rat, and Boström and Odeblad[22] observed that cortisone suppressed the ester $S^{35}O_4$ incorporation in chondroitin sulfate of rat skin. The mechanism of cortisone suppression of ester sulfate incorporation into the sulfomucopolysaccharide is unknown; presumably, the cortisone could effect the availability of polysaccharide precursors, the rates of synthesis, or release of the polysaccharides. It is also unknown if all the mucopolysaccharides, including hyaluronic acid, are similarly affected by cortisone.

The fibroblast and the synovial cell are thought to be cellular elaborators of hyaluronic acid. Cortisone could modify the function of these cells. The biologic effects of cortisone, hydrocortisone, and other steroids upon connective tissue vary considerably with the animal species. Wound healing, in which fibroblasts play an important role, is suppressed by small amounts of cortisone in man and the rabbit, while 50 mg. per kilogram is required to suppress wound healing in the guinea pig and the rat.[23]

In the present experiments with guinea pigs, cortisone, 30 mg. per kilogram daily, decreased the over-all synovial cell proliferation but not the number of small areas of polypoid proliferation due to intravenously injected bacterial polysaccharide. This effect upon synovial cells was accompanied by initial diminution and later increase in the presumed product of these cells, hyaluronic acid. Corticosterone did not reduce the over-all synovial proliferation but lessened the quantity of hyaluronic acid at seven days. The eventual explanation for such effects may be found in the rates of synthesis, cellular release, polymerization, and resorption of hyaluronic acid-complexes.

In the treatment of rheumatoid arthritis and related disease processes, cortisone and hydrocortisone are effective when given systemically and hydrocortisone is effective when given intra-articularly. After intra-articular injection of the steroid, the reduced viscosity and hyaluronic acid concentration of arthritic synovial fluid[24] tends to increase toward normal values.[25,26] The total protein concentration of synovial fluid shows no consistent change,[26,27] but the sodium ion concentration decreases toward the normal value.[27] Platt, Holley, and Pigman[28] found that although the clinical state is improved by such therapy, the synovial fluid does not return fully to normal, supporting the concept that the disease process has been suppressed but not cured. There may be a parallel between the steroid-treated joint disease in man and in the guinea pig: Corticotropin and cortisone increase the relative amount of hyaluronic acid without decreasing the protein material. This may be a significant clue in explaining the clinical improvement, if not the basic mechanism of the disease, in man.

The nature of the combination of hyaluronic acid and the proteins in synovial fluid has been the subject of controversy, and two types of union have been described: (*a*) the mucin precipitate formed by the addition of acetic acid and (*b*) the complex existing under physiologic conditions. The mucin-

precipitate formed by acidification at pH 5.5 or less is an artificial product which could not be expected to occur under physiologic conditions.[7] Since the first description of the mucin precipitate by Frerichs, in 1846, many studies on synovial fluid have been directed toward the composition or modification of this mucin.[19] The protein component is either predominantly albumin or a mixture of about equal parts of albumin and globulin.[11,30]

The naturally occurring complex of bovine synovial fluid has been investigated by ultrafiltration, by ultracentrifugation,[12,14,30] and by electrophoretic and immunologic techniques.[12] Ogston and Stanier [30] believe that the viscosity of the synovial fluid is dependent upon the existence and maintenance of a complex of approximately 70% hyaluronic acid and 30% protein. The protein component varies with the pH and ionic strength. At pH 5.8 and 7.0 the predominant protein is α-globulin, while at pH 8.0 γ-globulin is the major protein component.[12] Unlike the proteins of the acidified synovial fluid mucin, no albumin is present in the naturally occurring complex, but albumin is bound to the complex at pH 5.8 and 7.0 as the ionic strength of the buffer is increased. In the naturally occurring complex Curtain [12] suggests that the serum proteins may be adsorbed to the randomly coiled micelles of hyaluronic acid in a manner similar to the "salting-out adsorption" of proteins on solids observed by Tiselius. The physical properties of hyaluronate are also greatly effected by sodium concentration above 0.15 ionic strength.[31]

The hyaluronic acid-protein complexes in synovial fluid may have a relationship to the changes observed in the joints of the guinea pig. Hyaluronic acid as a hydrophilic, colloidal, anionic polyelectrolyte binds not only cationic proteins but other substances, including dyes. Some dyes by aggregation or polymerization develop a different spectral absorption, i. e., metachromasia. Sylven and Malmgren [32] state that the gel but not the sol state of hyaluronate is metachromatic. The binding of the dyes, like that of protein, will be affected by pH, electrolyte concentration, temperature, dielectric constant, etc.[33] Proteins, by competing with the dye for the anionic groups of the polysaccharide, reduce or even abolish the metachromasia.[33,34] The metachromatic property of synovial fluid is dependent upon the protein concentration; dialyzed native synovial fluid is nonmetachromatic but becomes metachromatic upon the addition of hyaluronic acid. Synovial mucin, with less protein than native synovial fluid, is intensely metachromatic; the addition of egg albumen abolishes this metachromasia.[34] Purified hyaluronic acid apparently is not PAS-reactive,[35,36] while commercial (40%) hyaluronic acid and synovial fluid are. The reason for this is not clear, but presumably involves the availability of 1,2-glycol or possibly the α-amino groups. Thus, the addition of protein seemingly results in the reduction of metachromasia and the appearance of PAS-reactivity of hyaluronate-containing preparations.

The fibrillar, finely granular, and occasionally homogeneous material in the guinea pig joints has the properties of hyaluronic acid (or hyaluronic acid plus some associated or combined proteins) in that it is metachromatic, is nonreactive with PAS, and disappears after incubation with testicular hyaluronidase. As with any histologic technique, the morphologic appearances may be, in part, artifactitious, for on smears the aspirated synovial fluid is more homogeneous. However, it is of interest that the width of the fine strands is not dissimilar in diameter from the mean radius of 140 mμ estimated for the coiled hyaluronic acid complex [14] and that linear or network aggregation of such particles might be expected to occur.[14]

The PAS-reactive granules, globules, and masses may be polysaccharide-protein complexes. With movement of the joint, the fine PAS-positive, orthochromatic granules occurring along the metachromatic fibrils could be molded into larger globular masses which are displaced or retained in the recess areas where they are most often seen. Some

polysaccharide content of the globules may be suggested by the PAS staining and occasional mild metachromasia. Further studies are obviously needed to determine the chemical composition of the different morphologic materials observed in the acute joint lesions of the guinea pig.

Exogenous steroid hormones could indirectly modify the joint lesions by alterations in capillary permeability, plasma proteins, and function of endocrine glands and other tissues and in the distribution and metabolism of the bacterial polysaccharide. That altered capillary permeability may play a role is suggested by the accumulation of mild quantities of protein-rich ascitic fluid after combined administration of corticotropin and cortisone for 10 days. The ascitic fluid is present in much greater volume if the bacterial polysaccharide has been injected intravenously some days before.[6] Such changes may have some relation to the differences in the effects of corticotropin plus cortisone and cortisone or corticotropin alone upon the eosinophilic PAS-positive material in the joints. Animals receiving the corticosterone disclose even more definite effects. Corticosterone alone produces a mild amount of PAS-reactive granules and globules in their joints, and the addition of the single intravenous injection of polysaccharide leads to "toxic" responses similar to those observed after acid-extracted polysaccharide (precipitate of bile in distended gallbladder, occasional death, intussusception, ascites, and engorged or hemorrhagic adrenals even after one week).[2]

Massive doses of exogenous steroids have markedly different effects in various animal species. These effects may be dependent upon the amount of steroids normally occurring in the species. In the present study, the most adverse effects were observed with corticosterone. Guinea pigs normally secrete hydrocortisone and very little corticosterone; the rat, on the other hand, is predominantly a corticosterone-secretor. As the guinea pig responds unfavorably to corticosterone, the rat is adversely effected by hydrocortisone; in each case the response is to a relatively "foreign" corticosteroid. Changes in the plasma proteins of the rat after the various administration of steroids and corticotropin have been described.[37] It is not known if similar plasma protein changes occur in the guinea pig or if such protein changes could alter the joint lesions which are induced by bacterial products. Conceivably, the relative amount of different plasma proteins diffusing into the joint space could modify the type and amount of protein combining with hyaluronic acid. Also, we have not investigated the significance of the dense clot which would sometimes form in heparinized plasma from animals receiving corticotropin plus cortisone with or without the polysaccharide. These clots may be related to the heparin-precipitable protein described by Thomas.[38]

Except for a marked decrease in the C^{14} uptake by the adrenal cortex in cortisone-pretreated animals,[6] no significant modifications in the distribution of the bacterial polysaccharide one week after injection have been found with the steroids or corticotropin used in the present study. In unpublished studies we have observed that plasma levels of C^{14} are much lower in cortisone-pretreated animals at two days after the injection of the labeled polysaccharide. Although the adrenals have a marked uptake in C^{14} from the labeled polysaccharide, they still produce 17-hydroxycorticosteroids. Marked elevation in free 17-hydroxycorticosteroids in bile and plasma occurs after the first and third daily intravenous injection of the K. pneumoniae polysaccharide complex.[39]

Summary

The acute arthritic lesions induced in the guinea pig by a single intravenous injection of a polysaccharide-complex from Klebsiella pneumoniae have been modified by corticotropin (ACTH) and certain steroids. Corticotropin and cortisone initially decrease and then increase the basophilic material having the histochemical characteristics of hyaluronic acid. The eosinophilic, PAS-

reactive material, apparently a complex of hyaluronic acid and protein, is not decreased by these hormones. Corticosterone initially increases, later decreases, the amount of "hyaluronic acid" as well as the eosinophilic, PAS-reactive substance. Progesterone, pregnenolone, 11-desoxy-17-hydroxycorticosterone, and vehicle controls did not significantly modify the joint change, but corticosterone (Compound B), a steroid relatively "foreign" to the guinea pig, leads to "toxic" responses to the nonlethal, nontoxic, haptenic bacterial polysaccharide.

The modification of the joint lesions is discussed in relation to (*a*) the effects of steroids upon connective tissue in general and upon joint disease in man and (*b*) the changes in the synovial fluid components and their histochemical characteristics.

Dr. Harry J. Robinson, Merck Institute for Therapeutic Research, supplied the corticosterone and vehicle No. 1; Dr. M. A. Schooley, The Armour Laboratories, contributed the corticotropin (ACTH), and Dr. André Robert, The Upjohn Company, contributed the desoxycorticosterone used in the present experiments.

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Chondrosarcoma of the Tongue

A Case Report

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The purpose of this report is to record a case of primary chondrosarcoma arising in the tongue. A survey of medical literature has failed to reveal any examples of such a tumor arising in this situation, although chondroma is rarely seen.[1] Recently, Stout and Verner[2] reported seven cases of chondrosarcoma arising in extraskeletal soft tissues and reviewed the literature on this subject; Stout[3] (who concurs with the diagnosis of this tumor) has not seen a chondrosarcoma in this location.

Report of Case

A 51-year-old man noticed a lump in his tongue three weeks before seeking treatment. He believed it was increasing in size. The tumor was superficially situated in the midportion of the left lateral border of the anterior tongue. Local excision of the tumor was undertaken six months ago. At the present time there is no clinical evidence of recurrent or metastatic tumor.

Grossly the mass was lobulated, apparently encapsulated, gray tissue, measuring 2 cm. in diameter and lying just beneath the mucosa. The cut surface was slightly cystic and gelatinous.

Microscopic examination revealed a well-differentiated chondrosarcoma composed of a primitive, cystic, cartilagenous matrix containing numerous atypical chondroblasts; the cells were round or stellate, moderately pleomorphic, with occasional multinucleated forms. Small nodules of tumor appeared to infiltrate and destroy adjacent striated muscle. There was no evidence of completely mature cartilagenous tissue or benign chondroma; furthermore, there was no indication of cartilagenous metaplasia developing in a different type of neoplastic process.

Submitted for publication Aug. 12, 1957.

Comment

From the distribution pattern of soft-tissue cartilagenous tumors, Stout and Verner concluded that the chondrosarcomas probably arose *de novo* and not from pre-existing benign chondromas. This appears to be true in the present case, although a small focus of benign chondroma or ectopic Meckel's cartilage could be rapidly overgrown and obliterated. If cartilagenous tumors arising in the anterior portion of the tongue developed from ectopic Meckel's cartilage, some concomitant malformation of the jaw might be expected; no history of this is evident from previous reports, however.

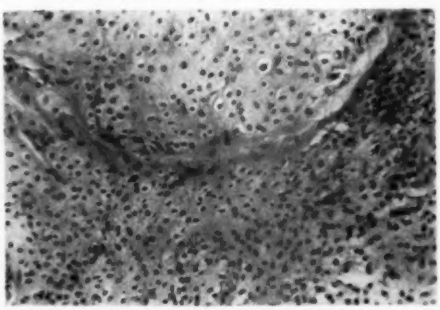

Figure 1

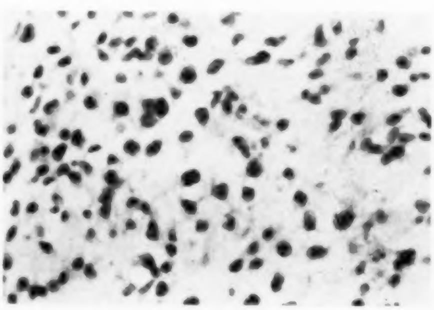

Figure 2

Conclusion

A primary chondrosarcoma arising in the anterior tongue is reported. It is believed to be the first such tumor to be recorded in this location.

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Study of the Air Content and State of Expansion of the Infant Lung

Reference to the Problem of Sudden Death

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It has been stressed repeatedly by many authors [1-7] that the most commonly affected system in cases of sudden death in infants is the respiratory tract. During the course of a study on the criteria of interstitial pneumonia,[8] observations were made which seemed to have a bearing on the problem of sudden and unexpected death in infants. The present report deals with the investigation of these findings.

Material and Methods

The lungs of an unselected series of 85 consecutively autopsied infants up to 2 years of age and stillborn infants after at least six months of intrauterine life were studied. Both lungs and part of the trachea were extracted *in toto* and processed as follows.

Venesection needles were inserted into the main bronchi of two lobes in each pair of lungs. The needles were ligated in position and connected to either a suction apparatus or a positive-pressure pump as shown in Diagram 1.

One lobe was inflated with air under a pressure of 100-150 mm. of mercury, and another lobe was deflated by a negative pressure of 300-400 mm. of mercury. The other three lobes were used as controls.

An equal number of the various lobes of the lungs were submitted to each procedure. In some cases, lobes which were to be deflated were tightly ligated across the middle, and the distal portions were removed. These portions were then submitted to different procedures so as to produce collapse. Some pieces were centrifuged in formalin at 3000 rpm for about 20 minutes. Others were compressed by a weight of 80 kg. All of the lobes and portions were then immersed in a 10% solution of formalin which was brought to 60 C by gentle warming so as to hasten fixation. The pressure in the bronchi of the two processed lobes was kept constant during the fixation. The formalin solution was then allowed to cool, and the lungs remained in the formalin for a further 24 hours.

Paraffin blocks were prepared, sectioned at 8μ, and stained with hematoxylin and eosin and Laidlaw's reticulum stain counterstained either by Van Gieson's method or with hematoxylin and eosin.

Submitted for publication July 25, 1957.

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A short account of this study was presented at the 19th Congress of the Medical Association of Israel, Jerusalem, March 12-15, 1957.

Normal Anatomy

The alveolar septum is known to consist of a capillary, ground substance, reticulum and elastic fibers, and most probably also a thin epithelial layer. In this study special attention was given to the three-dimensional arrangement of the reticulum fibers.

The average diameter of an alveolus in an apparently normal lung of a newborn

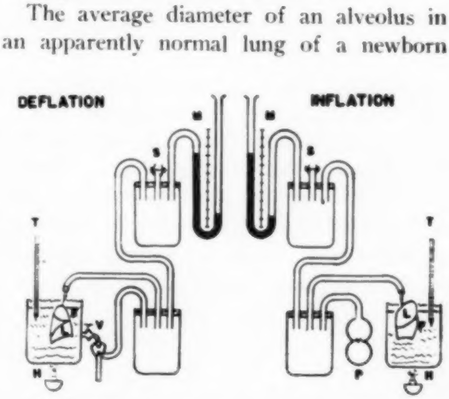


Diagram 1.—Apparatus used for the inflation and deflation procedures. Apparatus consists of two parts, an inflation and a deflation device. Each contains besides the pump also a mercury manometer (*M*), a bottle with two outlets, and a washbottle with three outlets connected to a venesection needle inserted into a bronchus. The lung is submerged in a beaker of warmed formalin solution.

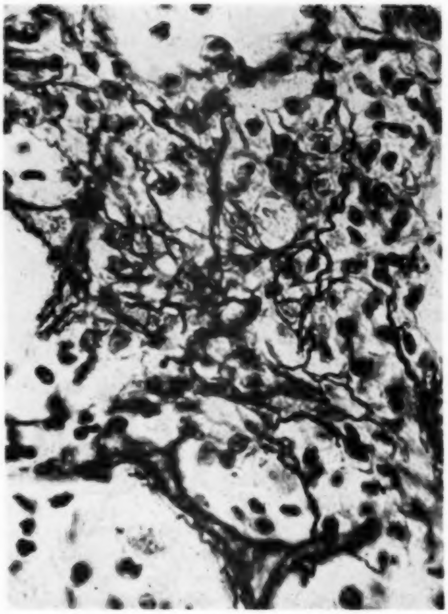


Fig. 1.—A transversely cut interalveolar septum. A mesh of reticulum fibrils surrounds individual cells. Laidlaw and Van Gieson; × 360.

infant who has lived for at least one day was found to be about 80μ. In a 2-year-old the diameter was about 115μ. These values are about 15% higher than those reported in the literature, probably because of the different techniques used, e. g., warming of the formalin solution.

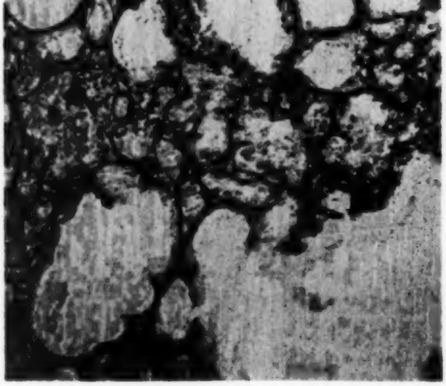
In many of our sections septa could be seen cut transversely. The chances of an interalveolar septum being cut tangentially depend on (*a*) the thickness of the sections, (*b*) the thickness of the septa, and (*c*) the alveolar diameter. This could be roughly expressed by the formula $C=X\frac{a+d}{2r}$, where *a* is the thickness of the section, *d*, the thickness of the septa, and $2r$ the alveolar diameter. The correcting factor necessary is represented by *X*, as the alveoli are not perfect hollow spheres, as the lung parenchyma is not made only of alveoli, and as the lumina are not surrounded on all sides by continuous alveolar walls.

Alveolar septa were studied in both transverse and cross sections. In the cross sections the reticulum fibers were found outside the capillaries and appeared as one or more slightly wavy threads. The number of the fibers often varied within the same septum, and in some areas short stretches without any reticulum were observed. This indicated that the reticulum fibers form a three-dimensional mesh in the alveolar septa, mainly around the capillaries. In the transverse sections of the septa the reticulum was seen as a dense network surrounding septal cells and anuclear plates (Fig. 1). In some areas the septa were cut obliquely and the appearance was intermediate between the two previously described pictures.

Findings in Nonmanipulated Lobes

In the study of the nonmanipulated lobes areas of overexpansion, varying greatly in size, were often seen. It was noted that these areas were usually adjacent to areas of compression (Fig. 2).

Fig. 2.—Adjoining areas of emphysema and atelectasis in an untreated lobe of an 11-day-old premature infant who suffered of incomplete aeration and died of bronchopneumonia. Laidlaw and Van Gieson; × 150.



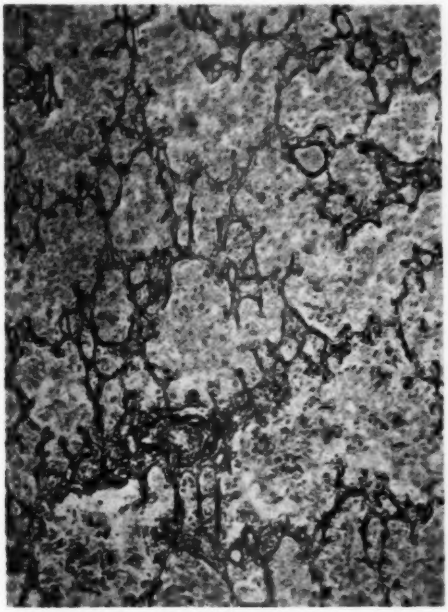


Fig. 3.—Overexpansion of alveoli by edema and amniotic fluid. Stillborn infant with evidence of severe aspiration of amniotic fluid. Atelectatic alveoli surround the overexpanded ones. Laidlaw and Van Gieson; × 150.

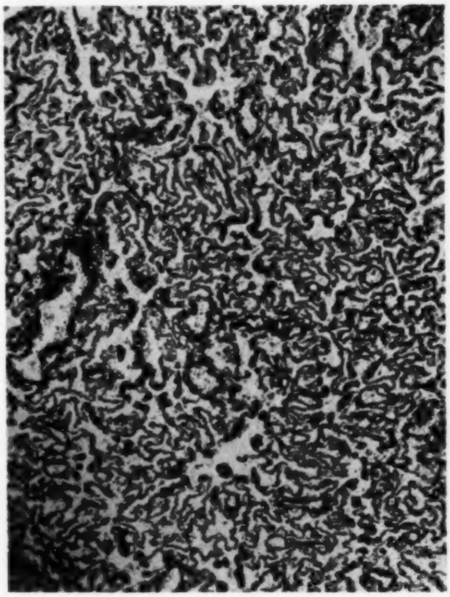


Fig. 4.—Diffuse atelectasis in an untreated lobe. Complete collapse of left lung in an infant suffering from acute tracheobronchitis. Wavy appearance of reticulum; septa appear thickened. Laidlaw and Van Gieson; × 110.

Areas with Increased Alveolar Volume (Overexpansion).—This was found in emphysema and also in other conditions where the alveoli were filled with blood, edema fluid, exudate, or amniotic fluid (Fig. 3).

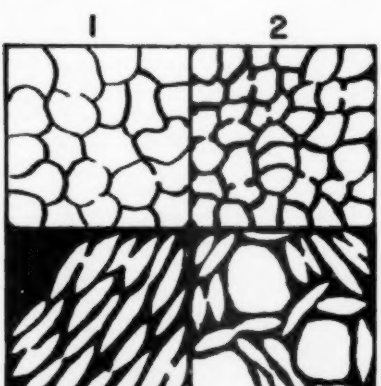


Diagram 2.—(*1*) Normal lung; (*2*) diffuse atelectasis; (*3*) polar atelectasis; (*4*) focal emphysema and microatelectasis (dysaeration).

The alveoli were distended, and the septa appeared thinner than usual. The reticulum fibers were stretched, and their waviness disappeared; there appeared to be fewer reticulum fibers in the septa, and in some places the fibers appeared to have ruptured, with slightly thickened and coiled ends. Fewer transverse septa were seen (see formula).

The overexpansion was not always general. In some cases it involved a whole lobe, in others (focal overexpansion) it involved only one alveolus or a group of alveoli.

Areas with Decreased Alveolar Volume. We confirmed that the alveoli of nonaerated lungs of newborn and stillborn infants have lumina.[9] Atelectasis (decreased alveolar volume) was found in the lobes of stillborn infants only in the cases where the parenchyma of one area was compressed by overexpansion of adjoining areas or by compression by a mass such as hernia or effusion.

Three different kinds of atelectasis could be distinguished in this material.

(*a*) Diffuse Type: This type of atelectasis involved whole lobes or lobuli of lung parenchyma. Its main distinguishing mark was that the atelectatic part was compressed on all sides. In these cases, the alveoli were smaller than usual and the septa appeared thicker (Diagram 2, Part *2*). Within the septa the capillaries were tortuous, and the reticulum fibers appeared wavy and coiled (Fig. 4). The diffuse type of atelectasis was found in obstruction of major bronchi and in the cases where the compressing mass was large.

(*b*) Polarized Atelectasis (Figs. 5 and 6): The alveoli appeared deformed with the long axis perpendicular to the line of the mechanical force (Diagram 2, Part *3*). In these cases most of the septa were not markedly thickened. Waviness of the reticulum fibers was noted only in the short stretches of the septa running parallel to the line of force. This occurred whenever the parenchyma was compressed, for example, when an overdistended area compressed the adjoining parenchyma against the pleura or a fibrous septum. Another type of polarized atelectasis was noted where the lines of force ran radially from a central space-occupying focus toward a periphery. In these cases the polar arrangement was concentric and not in parallel lines.

(*c*) Microatelectasis (Fig. 7): This type of atelectasis involved a small group of alveoli around a small focus of pressure. Thus, an overexpanded alveolus caused its neighbors to collapse (Diagram 2, Part *4*). Microatelectasis was also found between and around emphysematous alveoli, especially in lungs containing fluid or mucus in the airways.

The microatelectatic alveoli showed collapsed walls and a lumen which was often reduced to a narrow cleft. The reticulum within the septa was wavy, but less so than in diffuse atelectasis. It was often hard to distinguish microatelectatic alveoli from transverse sections of septa and from septa

Fig. 5.—Polar atelectasis in an untreated lobe due to compression of the tissue between an area of bullous emphysema and a fibrous septum. Hematoxylin and eosin; × 150.

Fig. 6.—The same area as in Figure 5. Reticulum fibers run mostly perpendicular to the lines of force. Laidlaw and Van Gieson; × 100.

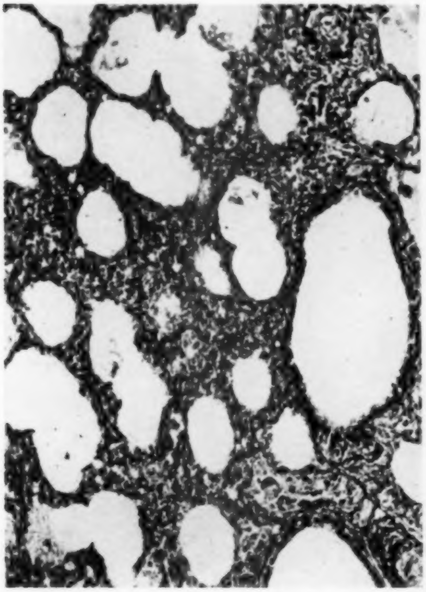


Fig. 7.—Microatelectasis around foci of emphysema. The apparently thickened septa consist of compressed alveoli. Laidlaw and hematoxylin and eosin; × 100.

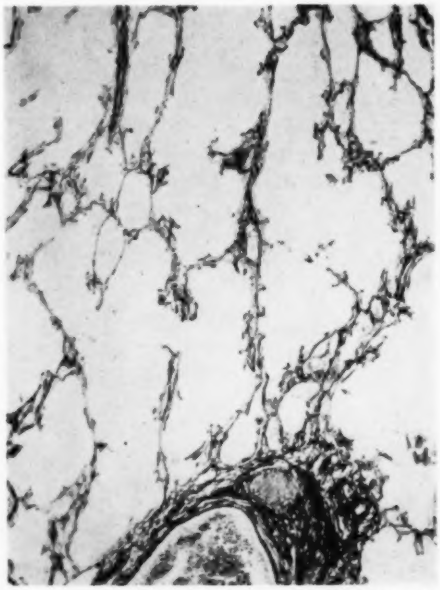


Fig. 8.—Artificially induced emphysema. Reticulum fibers are stretched and in places ruptured. Laidlaw and Van Gieson; × 90.

infiltrated by inflammatory cells, but microatelectatic alveoli could be recognized by their appearance in the reticulum-stained sections: The compressed cavity never contained any reticulum.

It should be noted that microatelectasis was found in a very large percentage of the cases examined (about 50%).

Findings in Manipulated Lobes

Overexpansion.—Overexpansion produced by inflation resulted in a picture similar to overexpansion in the nonmanipulated lobes (Fig. 8).

Decreased Alveolar Volume.—It was found that even the nonaerated lungs of newborns could often be collaped by aspirating some of the fluid content. While inflating lobes containing fluid, it was noted that unequal distribution of air occurred, resulting in foci of atelectasis and foci of overexpansion (Fig. 9) of the three different types of atelectasis.

(*a*) The Diffuse Type: This type was similar to that found in the nonmanipulated lobes.

(*b*) The Polarized Type: This type was best seen in pieces of living tissue compressed mechanically by centrifugation or by weight.

(*c*) Microatelectasis: Microatelectasis was found around overexpanded alveoli in artificially inflated lobes which contained fluid in the alveoli or the bronchial tree and also by artificial deflation of an emphysematous area (Fig. 10).

Comment

In this paper the terms "increased alveolar volume" or "overexpansion" were used rather than the term "emphysema." This was done as it was observed that emphysema, or increased air content, is a subgroup of the condition of overexpansion, as in many areas of increased alveolar volume the distended alveoli contained fluid instead of air.

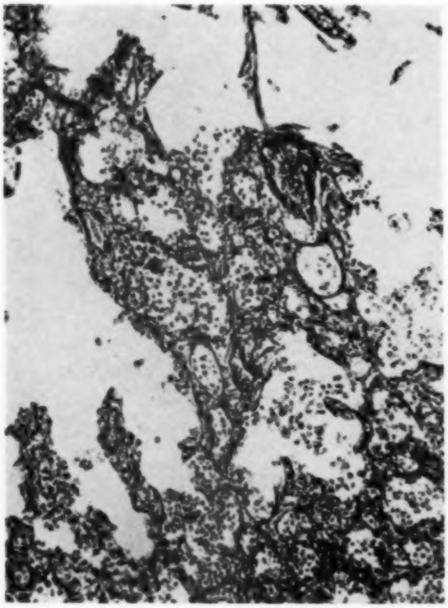


Fig. 9.—Adjoining areas of emphysema and atelectasis produced by artificial inflation. Laidlaw and Van Gieson; × 100.

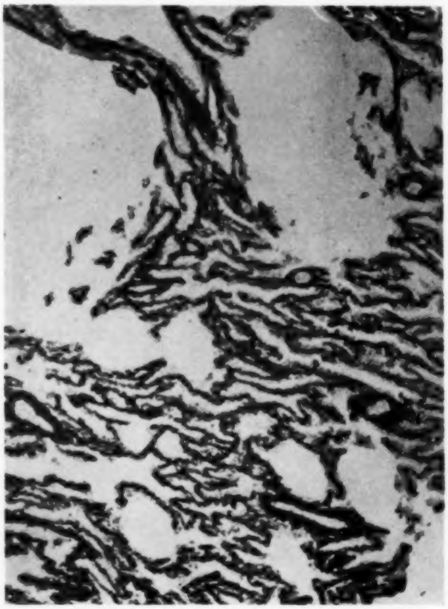


Fig. 10.—Microatelectasis around foci of bullous emphysema, produced by artificial deflation of an emphysematous lobe. The atelectasis is somewhat polar in arrangement. The reticulum is wavy. Laidlaw and Van Gieson; × 110.

In considering the problem of "fetal atelectasis" it was found that the alveoli of stillborn infants were of a given size and contained lumina filled with fluid (Figs. 11 and 12). In stillborn infants who died of anoxia during labor, the alveoli were overexpanded but contained amniotic fluid instead of air.[12,13] In the lungs of infants who had breathed only a few breaths, a few areas of marginal emphysema [10,11] were found. In the alveoli of infants who had lived for some days and died from a disease unconnected with the respiratory system the alveolar fluid was completely replaced by air and no areas of emphysema were seen. The difference in size between the alveoli of nonaerated and aerated lungs can be no more than that permitted by variations in volume of the thoracic cage. This can be no greater than the difference between forced expiration and the resting state.

Thus it is suggested that the lungs of stillborn infants should not be considered atelectatic, as the term atelectasis indicates collapse of tissue with decreased alveolar volume. An exception to this would of course be the condition caused by a space-occupying lesion in the chest. The term "fetal atelectasis" should be dropped and replaced by a more appropriate term, such as "nonaeration" or "atelectasis due to . . ." in the appropriate cases.

In our study three types of atelectasis were distinguished, two of which appear not to have been extensively studied before. The polarized atelectasis was found when a force compressed the lung parenchyma against a nondistensible structure. Microatelectasis was frequently observed in both the nonmanipulated and the manipulated lobes. The frequent production of microatelectasis by artificial inflation would seem to indicate its pathogenesis. An inflow of air into a lobe containing liquid within the bronchi causes some alveoli to expand, while others which have their bronchi plugged are compressed (Figs. 13 and 14). In the living lung this effect is probably enhanced, as trapped air

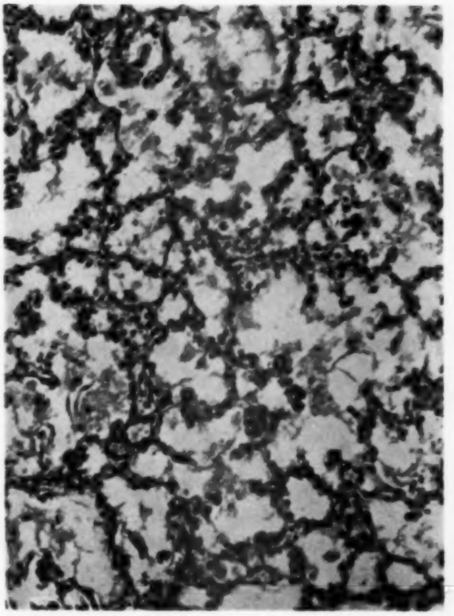


Fig. 11.—Nonaerated lung of a stillborn infant. The lumina of the alveoli are of normal size for the age and contain liquid and amniotic debris. Hematoxylin and eosin; × 150.

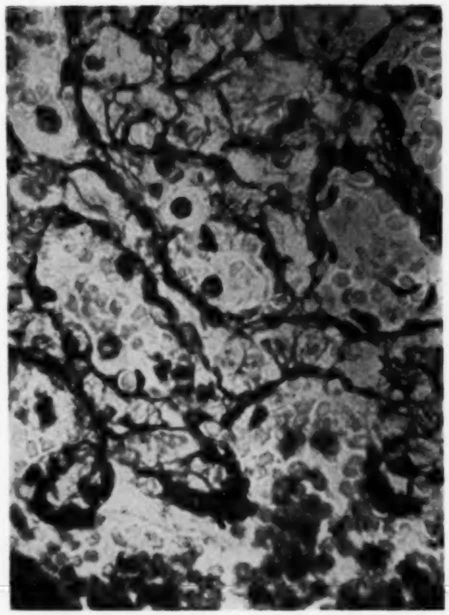


Fig. 12.—Nonaerated lung of a stillborn infant. The reticulum fibers do not show an increased waviness. Laidlaw and Van Gieson; × 560.

Fig. 13. Artificially produced focal emphysema and microatelectasis, produced by inflation of a lobe of an infant which suffered from bronchitis and bronchopneumonia. Few overexpanded alveoli surrounded by microatelectasis. Laidlaw and hematoxylin and eosin; × 110.

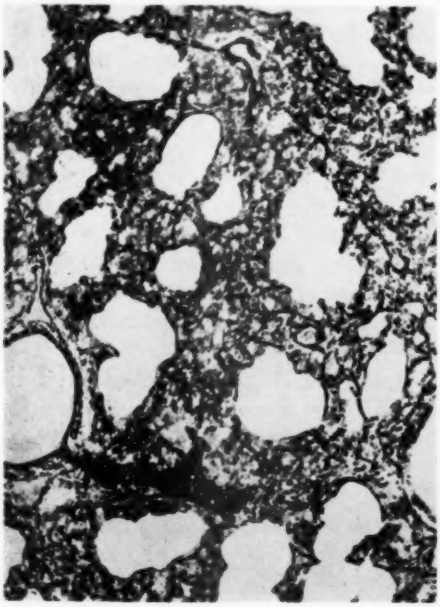
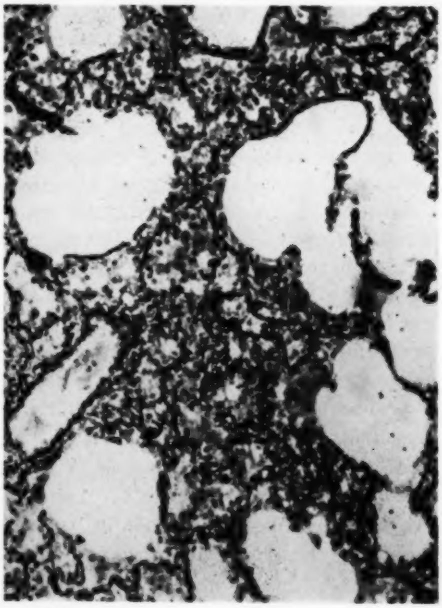


Fig. 14.—Naturally occurring focal emphysema and microatelectasis in an infant suffering meningo-encephalitis with pulmonary edema. Laidlaw and hematoxylin and eosin; × 150.



is rapidly absorbed. It is reasonable to assume that a similar mechanism might operate in some cases in vivo when fluid or droplets of mucus plug small bronchi or bronchioles. In infants a mild diffuse bronchitis, bronchiolitis, edema, or hemorrhage in the respiratory tract might start a vicious circle. Plugging of the air passages might cause hyperpnea, with resulting focal emphysema and microatelectasis. As both the emphysematous and microatelectatic changes in the alveoli reduce the exchange of gases, anoxia results with a further increase in the forceful respiratory movements. This leads to further emphysema and microatelectasis. This vicious circle might rapidly lead to sudden death. In some of these cases, postmortem examination may reveal insignificant changes in the respiratory tract (except for the unequal distribution of air), as reported by some authors.[3,6,13]

It was noted that the amount of focal emphysema and microatelectasis in the inflated lungs (which contained liquid in the bronchi) could occasionally be experimentally reduced by slow and repeated inflation and deflation. The change was probably due to the removal of fluid or mucus which was obstructing the air flow and an increased chance of uniform distribution of air through partially obstructed channels by a slower rate of flow.

The term dysaeration is proposed for the condition of focal emphysema surrounded by microatelectasis. This definition might correspond to the phenomenon of unequal ventilation of the pulmonary pathways described by physiologists.[14] It is reasonable to assume that the frequency of dysaeration in young infants and especially in premature newborn infants is due to the small caliber of the air passages in relation to the size of mucus droplets, desquamated epithelium, etc. The syndrome of congenital alveolar dysplasia [15,16] might also represent the same findings under another heading. The immature mesenchyme observed by the workers who described this condition probably represents the appearance of normal tangentially cut septa at this age. The appearance of tangential septa has been described by Engel, who remarked on the possibility of their being mistaken for enlarged or infiltrated alveolar walls.[17] Engel has also described the unequal distribution of air in the lungs of infants under the term "dystelectasis." It is noteworthy that the syndrome of congenital alveolar dysplasia was always found to be accompanied by the presence of (*a*) edema, (*b*) overdistention, and (*c*) compression of different alveoli. Similarly the syndrome of infantile lobar emphysema [18] might also represent dysaeration with preponderant focal emphysema.

The findings of this study might have a practical application in the treatment of newborn infants. It appears that the various drainage procedures in use might help to avoid the formation of dysaeration but that the administration of air or oxygen under pressure to infants is a dangerous procedure. In those cases where liquid or mucus is present anywhere along the respiratory tract this procedure might precipitate focal emphysema and microatelectasis. It is suggested that in infants suffering from hypoxia gas should be given, preferably accompanied by a recognized method of artificial respiration. The use of drugs to diminish hyperventilation might also be considered. The problem of rational mucolytic therapy effective in diminishing mucus secretion needs further investigation. Until then, drainage methods are probably the only answer to the removal of fluids from the respiratory tract.

Summary

The lungs of 85 infants up to the age of 2 years and of stillborn infants were studied. Various lobes were artificially inflated or deflated or nonmanipulated.

It was found that overexpansion of the alveoli is not always synonymous with emphysema. Overexpansion was caused in some instances by blood, edema, or other fluids.

Three different types of atelectasis were recognized: (*a*) diffuse, (*b*) polarized, and

(*c*) microatelectasis. Microatelectasis around areas of focal emphysema was commonly found in this series. The association of focal emphysema and microatelectasis was named dysaeration. Dysaeration was found to follow obstruction of air passages by exudate, mucus, or other fluids and forced inspiratory movements. It is suggested that through a vicious circle of anoxia, forced inspiration, and increased dysaeration this process may lead to sudden death in infants.

The findings of this study are considered to be relevant to the therapeutic and prophylactic approach toward anoxic states in infants.

Dr. H. Ungar, head of the Department, gave suggestions and Dr. H. Muhsam, from the Department of Statistics, gave advice in the preparation of this paper. Mrs. H. Weinman provided the microphotographs, and Mrs. Z. Perper gave technical assistance.

The Hebrew University-Hadassah Medical School.

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The Microscopical Criteria of Interstitial Pneumonia

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Interstitial pneumonia (I. P.) may be defined as an inflammatory process of the lungs with exudate within the alveolar septa. Whereas the nature of the process is clear and agreed upon, the histological criteria for establishing this diagnosis are apparently different in the various pathological laboratories. Many pathologists do not consider thickened septa containing histiocytic and mononuclear elements as evidence of I. P., whereas others regard the same feature as definite evidence of an interstitial inflammatory process. Thus, similar microscopical findings have been labelled by some authors [1-5] as interstitial pneumonia, by others [6,7] as congenital alveolar dysplasia, and by still others [8] as a form of atelectasis.

In this study the microscopical characteristics of interstitial pneumonia were investigated, and an attempt was made to establish criteria for differentiating this condition from other processes in which the alveolar septa appear thickened.

Material and Methods

The lungs of 85 unselected autopsied infants were studied. The procedure used has been described in the preceding article.[9] The inflation and deflation procedures were used in order to compare the appearances resulting from differences in air content with those produced by inflammatory conditions affecting the septa. In addition, 11 selected cases of clear-cut interstitial pneumonia of the "giant-cell" type or of the "plasma-cell" type were included, from which only paraffin blocks were available for study. These cases served as controls for testing the accuracy of the histological criteria of interstitial pneumonia which were derived from the study of the 85 cases.

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Results

Histological Characteristics of I. P.—In I. P. the interalveolar septa were thickened because of cellular infiltration, congestion, and edema. The process was either diffuse or focal. A special form of I. P which accompanied bronchitis and peribronchitis could be identified in some cases. In such cases the inflammatory infiltrate spread from the bronchial wall, probably along lymphatic channels and into the adjoining septa. The infiltrate in this form of pneumonia (lymphangitic pneumonia [10]) was usually composed of neutrophils. Furthermore, it was found that the reticulum fibers in the affected septa of these cases were often fragmented or had disappeared completely. Peribronchial pneumonia should be set apart from the other forms of I. P., as it is not a rare complication of bronchitis and as the infiltrate is usually different from that found in the other forms of I. P.

In most cases of I. P. the cellular infiltrate was composed of lymphoid cells with a variable proportion of plasma cells (Fig. 1) and of histiocytes. In some cases (so-called plasma-cell pneumonia) the plasma cells were distinctly predominant. In apparently more chronic cases the septa contained also fibroblasts and collagen fibers.

The appearance of the reticulum fibers was not similar in all cases. In most cases there were two wide bands of reticulum on both sides of the thickened septa, with a few connecting fibrils between them (Fig. 2). The cells of the infiltrate were situated between the bands and in most instances were not separated from each other by delicate fibrils (Fig. 3). The reticulum fibers did not appear to be wavy in the infiltrated septa. In most cases it seemed that the amount of reticulum in the thickened septa

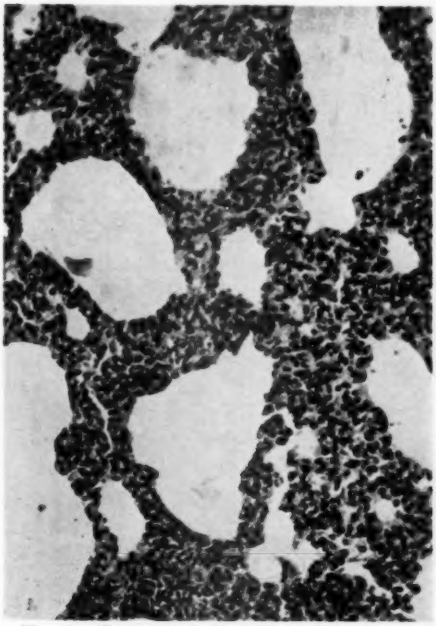


Fig. 1.—Focus of I. P. with mononuclear infiltration in the septa. Hematoxylin and eosin; × 150.

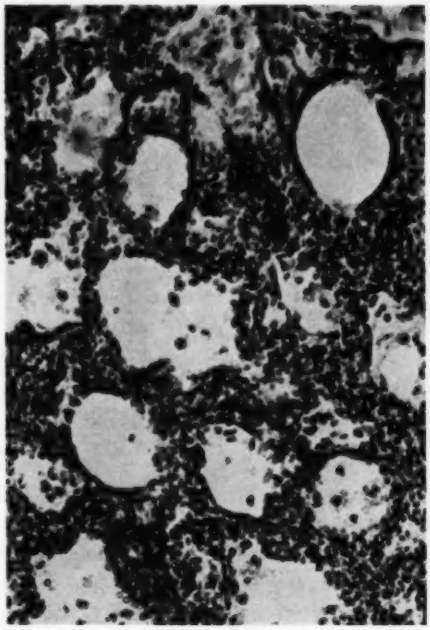


Fig. 2.—I. P. Thick bands of reticulum on both sides of infiltrated septa. Laidlaw and hematoxylin and eosin; × 150.

Fig. 3.—I. P. Thick bands of reticulum on both sides of a septum and more delicate fibers within the infiltrate. The fibers do not usually surround individual cells. Laidlaw and hematoxylin and eosin; × 530.

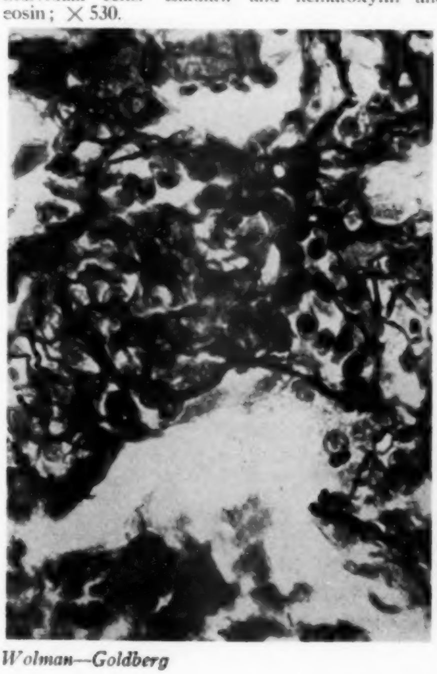
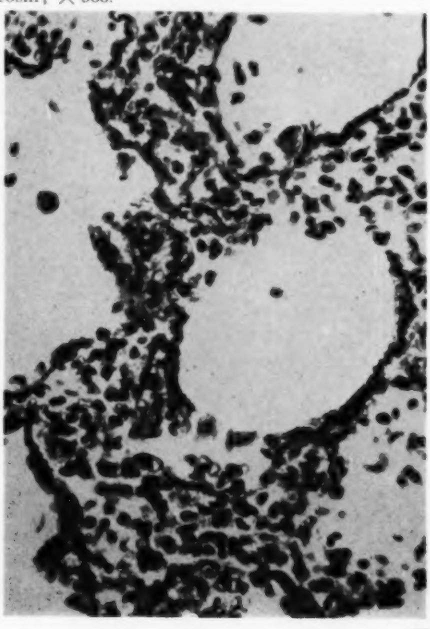


Fig. 4.—I. P. Thickened septa containing inflammatory infiltrate. Thickened peripheral reticulum fibers are torn in some places, completely missing in others. Laidlaw and hematoxylin and eosin; × 360.



was increased. This was indicated by the two thick peripheral bands and was even more prominent in the cases which were apparently more chronic and contained collagen and fibroblasts within the septa. In some cases there was evidence of disruption or destruction of the reticulum. The septa contained torn fibrils, often with coiled wavy ends, and many septa did not contain any reticulum (Fig. 4). A close association was noted between the appearance of disrupted reticulum fibers and the occurrence of pulmonary hemorrhages (Fig. 5). It is probable that an interstitial process which causes a dissolution of the fibers also erodes the vascular walls within the septa. It should be noted that in some cases in which the septa appeared thickened and apparently contained inflammatory cells the reticulum fibers were surrounded by the inflammatory cells. Close study of this finding showed that in these cases the inflammatory exudate was lying in the lumen and was adherent to the septa. This condition was probably caused by pressure of air on a semiliquid exudate and has nothing to do with I. P.

Fig. 5.—Parenchymatous hemorrhage bordering on a focus of I. P. Hematoxylin and eosin; × 100.

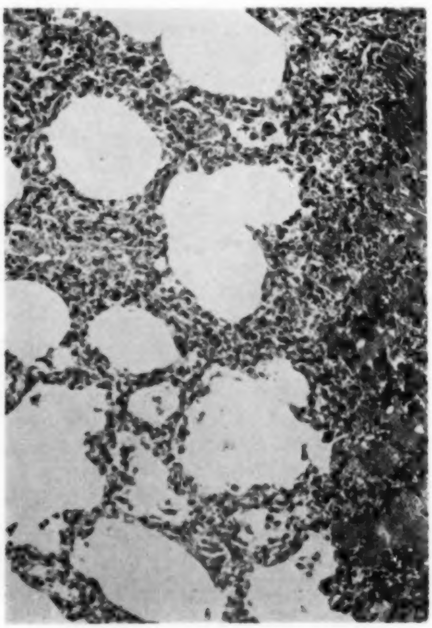


Fig. 6.—I. P. with emphysematous alveoli surrounded by microatelectatic ones. Laidlaw and Van Gieson; × 150.

In cases of I. P. the incidence of transversely cut septa was found to be increased. It may be pointed out that according to a previously published formula [9] the chances of seeing transverse septa increase in proportion to the thickness of the septa.

Microatelectasis [9] was found to be an almost constant accompanying feature of I. P. Inside and around the foci of I. P. there were microatelectatic alveoli and small emphysematous foci (Fig. 6). In other cases the foci of I. P. were found near areas of emphysema and atelectasis (Fig. 7).

In about half the cases of severe I. P. the lining of the alveoli was cuboidal in some areas (Fig. 8). In a few cases there was evidence of massive desquamation of these cells. In a few other cases giant cells of various types were noted.[11] In one case of this series interstitial edema was found. In this case the arrangement of the reticulum fibers was similar to that found in I. P.

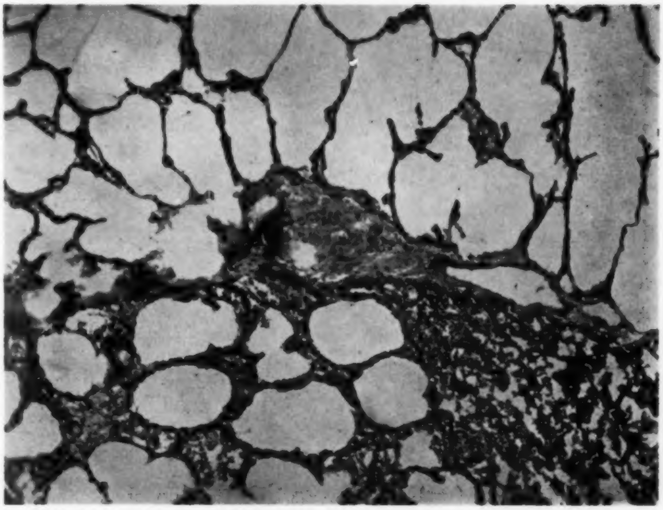


Fig. 7.—Focus of I. P. (on the lower left-hand corner) with emphysema in it, bordering on an area of frank emphysema (top) and on an area of diffuse atelectatis (lower right). Hematoxylin and eosin; reduced 8% from mag. × 90.

Effect of Inflation and Deflation on Lungs with I. P.—The effect of artificial inflation in areas affected by I. P. was similar to that obtained by natural emphysema. In both cases groups of alveoli were markedly enlarged, while the septa were still thick with the infiltrate present in them. Marked emphysema was associated with extreme distention of the reticulum fibers, which appeared like tight ropes and were torn in many places (Fig. 9). The thickening of the septa was easily recognizable in the emphysematous areas.

Deflation by any of the methods mentioned in the previous paper,[9] whenever successful, rendered the recognition of I. P. much harder, as the thickened septa were often approximated to an extent which rendered their outlines hardly discernible.

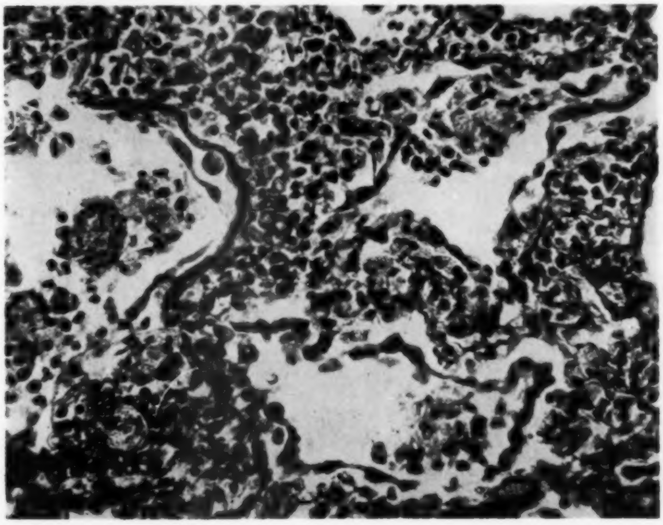


Fig. 8.—Focus of I. P. with cuboidal metaplasia of alveolar lining. Laidlaw and hematoxylin and eosin; reduced 8% from mag. × 340.

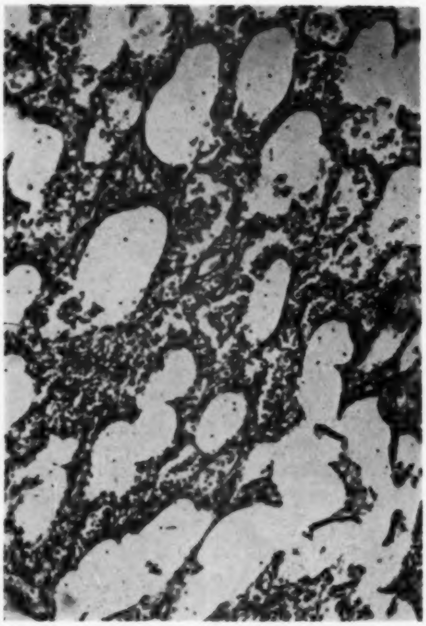


Fig. 9.—Effect of artificial inflation on a focus of I. P. Septa are thick with stretching and in places rupture of reticulum fibers. Laidlaw and Van Gieson; × 110.

Comment

The above-mentioned findings lead to the establishment of the following criteria for the microscopial diagnosis of I. P.

1. Thickening of the interalveolar septa by lymphoid, plasma-cell-like, or histiocytic and fibroblastic elements. In some cases, especially in the peribronchial type of pneumonia and in I. P. complicated by bronchopneumonia, the infiltrate may be rich in neutrophils.

2. Presence of reticulum fibers on both sides of the septal infiltrate. The reticulum fibers are present also in the infiltrate as delicate fibrils which do not surround the cells individually. The appearance of the reticulum fibers in most cases allows a clear differentiation of I. P. from microatelectasis on the one hand and from transversely lying septa on the other. Compressed alveoli can be recognized easily as they do not contain any reticulum in the lumen, whereas in transversely lying septa each cell is surrounded by the reticulum fibers. In most cases the reticulum fibers on both sides of the septa are thick. In some cases ruptured fibers are seen. This is never found in atelectasis.

3. The reticulum fibers are usually only slightly wavy in I. P. Increased tortuosity occurs only in the areas of microatelectasis which often accompany I. P.

Less constant features of I. P. are as follows.

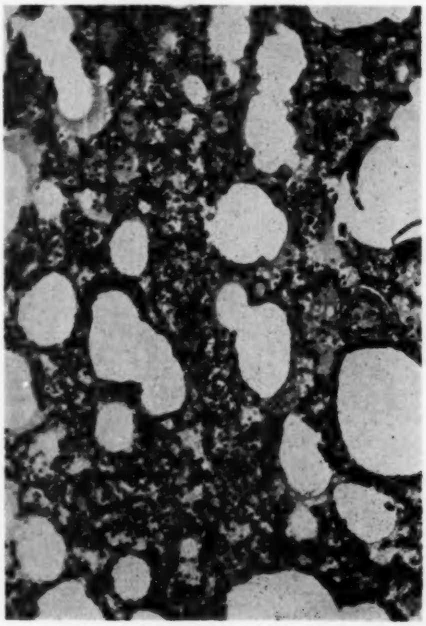
4. The presence of areas of focal emphysema and microatelectasis in and around the foci of I. P.

5. The presence of cuboidal metaplasia and desquamation of the alveolar lining. Occasionally, presence of giant cells.

6. The presence of parenchymatous hemorrhages.

A review of the literature indicates that some authors reported an extremely high

Fig. 10.—Lung of a 7-month-old infant who died after an operation for hygroma colli. Edema with focal emphysema and microatelectasis. Cursory examination gives the impression of thickened septa, but on careful observation the "septa" are seen to consist of microatelectatic alveoli. Hematoxylin and eosin; × 100.



incidence of I. P. among infants, perhaps because of the assumption that thickened septa indicated an interstitial inflammatory process.[1-5] It seems obvious that some of the cases regarded as I. P. belonged to the group described in a previous paper.[9] In this group the thickening of the septa was due to microatelectasis around foci of emphysema and not to an inflammatory process of the lung parenchyma (Fig. 10).

It appears that I. P. as seen in postmortem material is a subacute or chronic inflammatory process. The present findings do not give a definite clue to the sequence of events, but on theoretical grounds it seems probable that rupture of reticulum fibers and hemorrhage indicate a fulminating process. On the other hand, marked increase and thickening of those fibers as well as invasion of the septa by fibroblasts and collagen probably denote a more chronic process. By analogy with other pathological changes in the lungs it seems that metaplasia of the alveolar lining in infants might also belong to the chronic phase of the disease. These conclusions indicate that I. P. might progress from an exudative stage to a fibrosing one and result in fibrosis of the lungs. It cannot be ruled out, however, that "interstitial" infiltrations might also result from the organization of atelectatic infiltrated alveoli, assuming an appearance which cannot be distinguished from I. P.

One of the major puzzles in the clinical course of I. P. is the sudden unexpected death occurring in many cases, especially in young and premature infants. It is believed that sudden death in many cases of I. P. depends on the mechanism outlined in the preceding paper, that is, the disturbed oxygenation of the blood (caused by the increased distance between septal capillary and the lumen of the alveolus) which results in forced respirations. These respirations and the presence of mucus droplets (or exudate, or exfoliation) in the bronchi cause an unequal distribution of air and faulty gas exchange, with focal emphysema and microatelectasis (dysaeration[9]). The vicious circle of anoxia, forced respiration, focal emphysema, and microatelectasis might account for the sudden deaths. Sudden death might also be caused by extensive focal parenchymatous hemorrhages[12] from ruptured blood vessels in the inflamed septa.

Summary

A study was made of the lungs of an unselected series of 85 infants up to the age of 2 years and of 11 unquestionable cases of interstitial pneumonia (I. P.). In the 85 cases one lobe was artificially inflated, another was deflated, and the remaining lobes were not manipulated.

The histological characteristics of I. P. were investigated, and criteria for the histological diagnosis of this disease were established. These include (*a*) thickening of alveolar septa by a chronic inflammatory exudate; (*b*) presence of reticulum fibers on both sides of the septa, with delicate fibrils within the septa; (*c*) normal waviness of the reticulum fibers; (*d*) focal emphysema and microatelectasis in and around the foci of I. P. In addition, cuboidal metaplasia of the alveolar lining and parenchymatous hemorrhages are often seen.

It was found that I. P. may be associated either with new formation or with destruction of the reticulum fibers. The destruction is often associated with hemorrhages.

Sudden and unexpected death in many cases of I. P. is considered to be due to dysaeration.

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Effects of Ethionine Administration in Rabbits and Dogs

I. Changes in Serum Proteins, Lipids, Lipoproteins, and Glycoproteins and in Blood Coagulation

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Hyperlipemia during acute abdominal crises of acute pancreatitis was observed as early as 1846.[1] Recently there has been increased interest in this field because of observations indicating that familial hyperlipemia may represent a genetically transmitted predisposing factor for relapsing pancreatitis,[2,3] although this concept is still debatable.[4] It appeared profitable, therefore, to study experimentally the relations between pancreatitis and alterations of blood lipids in animals.

Various groups of investigators described destruction of acinous cells of the pancreas in animals following the administration of ethionine.[5-11] This substance was then employed to study the effect of pancreatitis on serum lipids. A pilot study showed that the administration of ethionine to rabbits resulted in pancreatitis and hyperlipemia.[12] Duodenal hemorrhage was noted on several occasions. This study presents a detailed report on the manifold effects of ethionine in rabbits and dogs.

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Material and Method

Rabbits.—Nineteen "gray chinchilla" rabbits, 15 males and 4 females, average weight 3.3 kg., were given daily injections of ethionine by the intraperitoneal route. A 2.5% solution of ethionine in saline was used in varying quantities. Of the 15 males, 2 received 500 mg. of ethionine daily for 7 days; 9 received 300 mg. daily, 5 of these for 4 days and the other 4 for 10 days. The remaining four animals received 100 mg. daily, two of these for 6 days and two for 10 days. The four female animals given 200 mg. of ethionine intraperitoneally daily died after four and seven days. Pathological studies on these animals will be presented elsewhere. Because of the increased susceptibility of the females, only male animals were used for these studies. During the entire observation period the animals received Purina Rabbit Chow and water ad libitum.

Blood was drawn from the marginal ear vein before and at various intervals during the administration of ethionine. The following determinations were performed on each specimen. Serum cholesterol, phospholipids, and total lipids were analyzed by the standard methods used in this laboratory.[13-15] In addition, blood sugar,[16] serum protein,[17] and serum amylase[18] were determined. The patterns of serum proteins, lipoproteins, and glycoproteins were studied by paper electrophoresis,[19] with use of amido-Schwartz for the staining of protein, oil red O for lipids, and the periodic acid-Schiff reagent for glycoproteins.[20] In the four animals receiving 100 mg. of ethionine daily serial determinations of blood coagulation and one-step prothrombin time[21] were carried out.

In two animals given 300 mg. of ethionine daily for 10 days the studies were performed prior to and during ethionine administration as well as 9 and 18 days after its discontinuation. All of the other animals were killed with pentobarbital (Nembutal) approximately 24 hours after the last injection of ethionine. Postmortem studies were performed in all animals.

Dogs.—Five male mongrel dogs whose body weight varied from 5.7 to 15.0 kg. were given ethionine, 100 mg. per kilogram in a 2.5% solution in saline, intraperitoneally. The injections were given every other day for 11 to 17 days. Blood was drawn from the jugular vein at weekly intervals. The specimens were subjected to the same chemical and electrophoretic studies as were performed in the rabbits. The dogs were fed once a day a diet consisting of horse meat, vegetables, and milk. Water was given ad libitum.

In three dogs serial determination of coagulation time, prothrombin time, and hemoglobin were performed at zero and four hours and then at intervals of one to three days after the administration of ethionine. Fibrinogen levels and platelet count were determined sporadically.

All animals were killed with pentobarbital, and postmortem studies were performed.

Results

Rabbits.—Ethionine produced in all doses used severe systemic changes shown mainly by pronounced anorexia, marked loss of weight, and emaciation. Females appeared to be more susceptible to the toxic changes than males. The serum lipids increased markedly four and seven days after the administration of large doses of ethionine (500 mg.) per day. The sera became lipemic (Table 1). With medium daily doses (300 mg.), however, there was a uniform depression of serum lipids in the first 4 days, followed by a rise after 10 days of treatment. At this time, the sera were slightly lipemic. The small daily doses used (100 mg.) produced a progressive depression of all serum lipid fractions to very low levels. The blood sugar decreased in all three groups of animals. The serum amylase showed a slight but not significant rise.

Serum total protein decreased after the administration of ethionine. This was evident both by chemical analysis and by paper electrophoresis (Table 2). All of the serum protein components stained faintly with amido-Black (Fig. 1). Nevertheless, the relative amount of albumin was increased. This relative increase of albumin was associated with slight decreases of β- and γ-globulins. The serum lipoproteins and glycoproteins decreased markedly. It was,

TABLE 1.—*Effect of Ethionine Administration on Serum Lipids, Blood Sugar, and Serum Amylase Levels in Rabbits and Dogs**

Animals, No.	Body Wt., Kg.		Before Treatment					Ethionine I.P.			4 Days					7-10 Days				
	Initial	Final	Chol. T/E, Mg./100 Cc.	P.L. Mg./100 Cc.	T.L., Mg./100 Cc.	Sugar, Mg./100 Cc.	Amylase, Units	Dose, Mg./Day	Duration, Days	Total Dose, Gm.	Chol. T/E, Mg./100 Cc.	P.L., Mg./100 Cc.	T.L., Mg./100 Cc.	Sugar, Mg./100 Cc.	Amylase, Units	Chol. T/E, Mg./100 Cc.	P.L., Mg./100 Cc.	T.L., Mg./100 Cc.	Sugar, Mg./100 Cc.	Amylase, Units
									Rabbits											
2	3.4	2.9	58/42	136	405	100	..	500	7	3.5	131/35	260	1930	84	..	120/64	192	565	66	..
9 †	3.2	2.8	57/43	142	368	108	200	300	4-10	1.2-3.0	41/25	132	324	62	305	202/125	244	943	73	299
4	3.6	3.2	58/40	135	460	106	169	100	6-10	0.6-1.0	34/24	110	240	114	209	13/8	61	113	56	301
Normal Range ‡						82-136	133-263													
									Dogs											
											7 Days					11-14 Days §				
5	10.8	9.0	210/163	430	875	60	295	0.6-1.19 ‖	11-17	3.4-12.0	186/136	334	689	69	270	93/77	245	348	62	293
Normal range ‡						50-75	150-400													

* I. P. indicates intraperitoneally; Chol. T/E, cholesterol total/esterified; P. L., phospholipids; T. L., total lipids.

† In 2 rabbits of this group serum lipids were studied 9 and 18 days after the discontinuation of ethionine administration. The average values were as follows: Chol. T/E, 64/48; P. L., 129; T. L., 420; sugar, 155, and amylase, 303.

‡ Normal ranges are based on the determinations on 20 normal animals.

§ Further chemical analyses in one dog which survived ethionine treatment for 17 days showed the following values: Chol. T/E, 62/54; P. L., 100; T. L., 220; sugar, 62 and amylase, 516.

‖ Ethionine, 100 mg. per kilogram of body weight intraperitoneally every other day.

TABLE 2.—*Effect of Ethionine on Serum Protein, Lipoproteins, and Glycoproteins*

	Before Ethionine Treatment						After Ethionine Treatment (10-14 Days)					
	Amido-Schwartz-Stainable Fractions, %											
Proteins	Total, Gm./100 Cc.	Alb.	α_1	α_2	β	γ	Total, Gm./100 Cc.	Alb.	α_1	α_2	β	γ
Rabbits	6.4	46.5	12.0		18.8	22.8	5.5	53.9	12.7		16.3	17.1
Dogs	6.8	69.5	3.9	5.9	5.8	14.9	5.2	82.0	1.5	4.1	9.1	3.3
Lipoproteins	Oil-Red-O-Stainable Fractions, %											
				α	β plus O			α	β plus O			
Rabbits				35.2	64.8			20.3	79.7			
Dogs				68.5	31.5			*	*			
Glycoproteins	Periodic Acid-Schiff-Positive Fractions, %											
			α_1	α_2	β			α_1	α_2		β	
Rabbits			26.7	56.0	17.3			26.5	56.0		17.5	
Dogs			18.5	58.0	23.5			17.0	58.0		25.0	

* No stainable lipids were obtained on repeated trials.

therefore, necessary to use two to four times the usual amount of serum for the separation of these components by paper electrophoresis. A relative decrease of α-lipoprotein and an increase of β plus O fraction were found. There were no appreciable changes in the relative concentrations of the various glycoprotein fractions.

After discontinuation of ethionine administration in two rabbits, serum lipids returned after 9-18 days to levels similar to those observed before ethionine administration (footnote to Table 1). Serum proteins, lipoproteins, and glycoproteins studied by paper electrophoresis also returned toward the original patterns.

The three-tube coagulation time increased rapidly from a normal of 4½ minutes to 6, 18, and over 30 minutes on the 6th, 9th, and 11th days of administration of 100 mg. of ethionine daily in four rabbits (Fig. 2). The prothrombin time increased from a

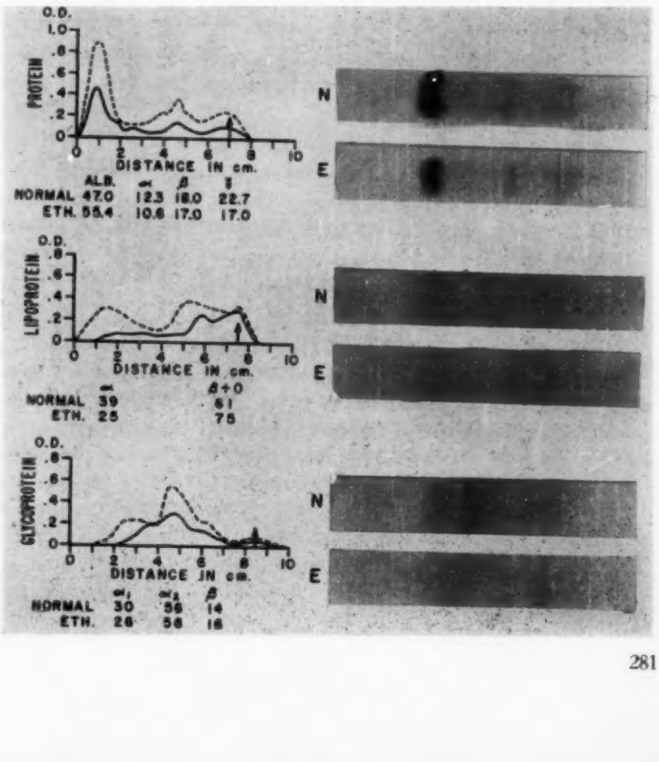

Fig. 1.—Serum proteins, lipoproteins, and glycoproteins separated by paper electrophoresis (rabbit). The stained paper strips are seen in the right half of the figure, and the planimetric graphs, in the left half. The upper strip of each series (*N*) represents the analysis before ethionine administration; the corresponding planimetric curve is drawn as interrupted line. The lower strip of each series (*E*) represents analysis after 10 days of ethionine administration (100 mg. daily by the intraperitoneal route); the corresponding planimetric curve is drawn as solid line. Note marked reduction of all serum components with the exception of the relative amount of serum albumin (see text).

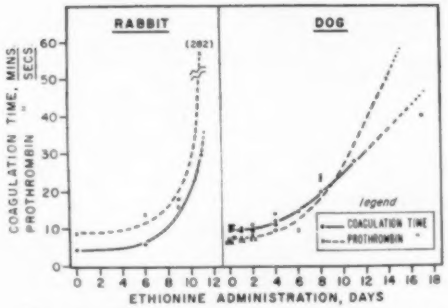

Fig. 2.—Effect of ethionine administration on coagulation time and prothrombin time in the rabbits and in the dogs. (For details see text.)

normal of 9 seconds to 14, 16, and 282 seconds on the corresponding days.

Dogs.—All dogs showed anorexia, weakness, and loss of weight after five to seven days of ethionine administration. During the second week, the general condition of the animals deteriorated rapidly, resulting in progressive cachexia. The urine contained bilirubin. The serum and the sclerae became icteric. Progressive hemoconcentration and a profound defect in blood coagulation interfered greatly with the drawing of blood specimens and with the subsequent chemical analyses. These blood samples remained liquid, and no separation of serum could be obtained on repeated centrifugation (fibrinolysis).

While the levels of serum lipids fell in all instances (Table 1, last line) no appreciable change was noted in blood sugar or serum amylase levels. Two animals had terminally undetectable amylase levels. This drop of amylase was also demonstrated by others.[22]

Serum proteins decreased markedly following the administration of ethionine (Table 2). By paper electrophoresis the relative amount of albumin was, however, increased. This increase was accompanied by decreases of α_{-1}, α_{-2}, and γ fractions, especially pronounced for γ-globulin. The β fraction was relatively increased. The lipoprotein fractions were not stainable with oil red O, despite the use of larger quantities of serum (two to four times the usual amount). The possibility of some radical changes in the lipoprotein molecules had to be considered. The total amounts of periodic acid-Schiff-positive substance in the serum were also decreased. With the application of four times the usual amount of serum the percentages of the various glycoprotein fractions did not change.

The three-tube coagulation time was prolonged progressively after the fourth day of ethionine administration. It increased rapidly from an average normal of 9½ minutes to 11½, 14½, 20, 28, and 40 minutes after 4, 6, 8, 11, and 17 days of ethionine administration, respectively (Fig. 2). Failure in separation of serum, resembling fibrinolysis in postmortem blood, after 11 to 13 days of ethionine administration made determination of coagulation time impossible.

Prolongation of prothrombin time was first noted after six days of ethionine administration. It increased from an average normal of 7 seconds to 9, 23, and 50 seconds after 6, 8, and 14 days, respectively. Again, hemoconcentration and fibrinolysis interfered with further determinations. The prolongation of prothrombin time could be corrected to normal by adding to the blood of ethionine-treated animals 1/10 vol. of normal plasma. However, it failed to return to normal if the normal dog plasma had been deprothrombinized with barium sulfate, indicating a deficiency in plasma prothrombin.

Platelet counts were reduced to 100,000 and 42,000 after 8 and 15 days, respectively, of ethionine administration (control level of 420,000 per cubic millimeter).

Comment

Earlier workers have shown that in rats ethionine inhibited the incorporation of labeled glycine and methionine into protein[23] and produced fatty livers.[24] Subsequent studies showed that prolonged administration of ethionine to dogs resulted in almost complete disappearance of circulating lipids and lipoproteins determined by the ultracentrifuge method.[25] It was thought that the effect of ethionine on lipid metabo-

lism was secondary to the impaired protein synthesis.

The results of the present study are, as a whole, in good accord with these observations. However, ethionine administered in large doses (300-500 mg. per day) produced in rabbits transient lactescence of the serum and elevation of all lipid fractions. The highest dose used resulted in earlier elevation of circulating lipids. It may be assumed that the inhibition of protein synthesis mentioned above resulted in rapid mobilization of lipids from the depots and elevation of all lipid fractions (transport lipemia).[26] The lowering of all plasma lipids after prolonged use of ethionine would be the result of inhibited lipid synthesis, as often encountered in states of emaciation and cachexia. It is of interest that the changes in serum lipids, as well as those of serum protein and lipoproteins, proved to be reversible within the limits of the experimental procedure employed.

Pathological observation to be reported later revealed hypertrophy of the adrenal cortex in most of the animals treated with ethionine longer than six days. These changes of the adrenals were perhaps in causal relationship with the changes of plasma lipids. The role of the adrenal cortex in the control of serum lipids has been the subject of repeated studies.[27] Whereas the elevation of serum lipids could be explained on this basis, the eventual decrease and/or disappearance of the circulating lipids may be interpreted as the result of adrenal cortical exhaustion.

The failure of oil red O to stain lipoproteins separated by paper electrophoresis presented another interesting observation on the effect of ethionine administration. In addition to the quantitative changes in circulating lipids determined chemically, probably a major alteration in serum lipoproteins took place. Whether this change is secondary to the alteration of serum proteins or independent of it requires further investigation. Correlative studies of plasma proteins and lipoproteins with the ultracentrifuge or the Cohn fractionation technique would be of great value in this respect.

Disturbances in blood coagulation in both rabbits and dogs are another facet of the extensive damage produced by ethionine. Deficiency in prothrombin may be the result of hepatic damage. The normal fibrinogen level does not necessarily imply intact hepatic function. Plasma fibrinogen is usually elevated in mild hepatic damage and depressed in overwhelming hepatic failure. Prolonged coagulation time after ethionine had been noted in rats [28] and guinea pigs.[29] In addition to coagulation defects, marked depression of plasma antihemophilic globulin and severe depression of the bone marrow was observed in the rat.[30]

As a powerful blocking agent of protein synthesis ethionine depressed all factors essential for blood coagulation. In the present study a hitherto undescribed phenomenon resembling fibrinolysis was observed. Although the mechanism and significance of fibrinolysis is poorly understood, evidence is accumulating that fibrinolytic activity in vivo can be induced by acute stress.[31]

It is evident that the effects of ethionine were very extensive and complex. They were caused by severe pathological changes in many vital organs, as will be shown in a subsequent paper. Inhibition of protein synthesis was associated with complex disturbances in serum lipids, lipoprotein, and glycoproteins and in the factors involved in blood coagulation. In view of these changes the hyperlipemia observed after ethionine administration is probably caused by a multitude of factors. The role of the pancreas in this mechanism cannot be delineated at present. A more specific technique producing pancreatitis without the associated severe changes in other organs would be advisable. Ethionine, because of its toxicity, is not suitable for the study of the relationship between pancreatitis and serum lipids.

Summary and Conclusions

Ethionine in doses ranging from 100 to 500 mg. per day was given intraperitoneally

to 19 rabbits (15 male and 4 female) and in doses of 100 mg. per kilogram every other day to 5 male dogs. Female rabbits seemed to be more susceptible to the toxic effect of ethionine than males.

Ethionine produced a progressive drop in serum proteins, lipids, lipoproteins, and glycoproteins. In rabbits treated with larger doses of ethionine serum lipids showed transient elevation followed by subsequent diminution. The possible association of these changes with initial stimulation and eventual exhaustion of the adrenal cortex is discussed.

A defect of blood coagulation was demonstrated in rabbits and dogs after ethionine administration.

Ethionine produced extensive and profound disturbances in protein and lipid metabolism. The damage was widespread and involved many vital organs. Ethionine proved to be too toxic for the specific study of experimental pancreatitis and its relationship to serum lipids.

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Effects of Ethionine Administration in Rabbits and Dogs

II. Pathological Studies

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In a previous report the effect of ethionine on serum proteins, lipids, glycoproteins, and lipoproteins and on blood coagulation was described.[1] Ethionine was found to produce not only profound metabolic disturbances but also structural alterations in various organs, which will be the subject of this report. The experimental procedure and the doses of ethionine employed were described previously. The organs involved are presented in the order of frequency or severity of the pathological changes. Attempts were made to correlate the structural and biochemical changes.

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Gross and Microscopic Findings

Liver.—The earliest changes were seen in the livers of rabbits and dogs. They consisted of marked fatty infiltration. This was present in all the experimental animals, even those given the smallest dose of ethionine (100 mg. daily for four days). Grossly the color of the liver appeared amber to yellow. Punctate hemorrhages were occasionally noted within the parenchyma. Microscopically, the liver cells were enlarged and contained considerable numbers of vacuoles (Fig. 1). In frozen sections the vacuoles were stained red by Sudan IV. The fat infiltration of the parenchymal cells was

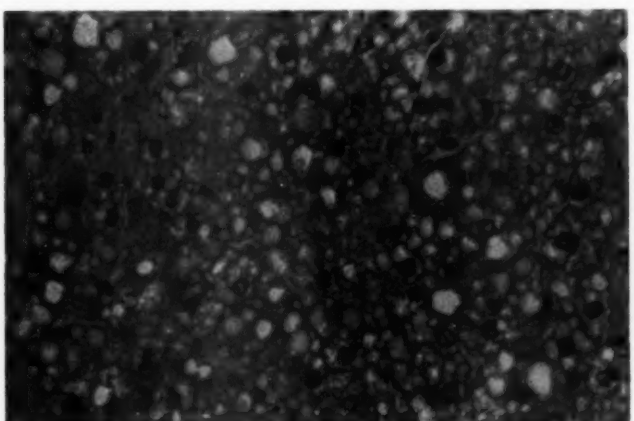

Fig. 1.—Liver of Rabbit 540, given daily intraperitoneal injections of 300 mg. of ethionine for 10 days. Note extensive fatty infiltration of liver cells. Hematoxylin and eosin; enlarged four times from mag. × 212.

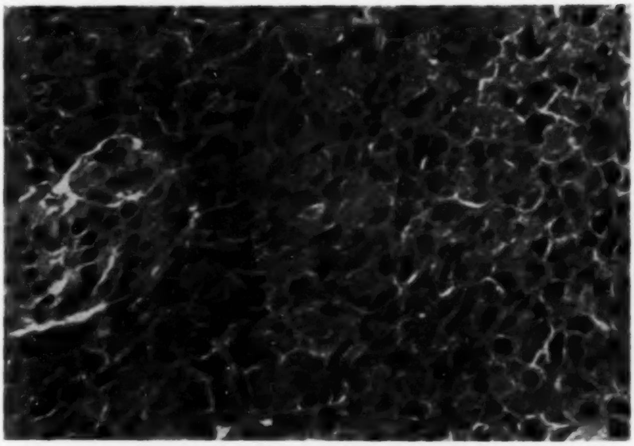

Fig. 2A.—Pancreas of an untreated rabbit. One islet of Langerhans is shown on the left side. Hematoxylin and eosin; enlarged four times from mag. × 212.

most marked in the peripheral part of the hepatic lobules. When the doses of ethionine were increased (500 mg. daily for seven days), the changes in the liver became severer. Not infrequently, necrosis of parenchymal cells and infiltration with polymorphonuclear leukocytes were encountered. In four rabbits, there was early focal ductular transformation of liver cells in the periphery of the hepatic lobules.

In one animal, hemorrhage into the wall of a small bile duct was noted. The latter was considered to be a part of the hemorrhagic manifestations seen in ethionine-treated animals.

Pancreas.—In all the experimental rabbits the pancreas was edematous, pale, and firmer than normal. In four, hemorrhagic areas were noted. Gross fat necrosis was seldom seen. Microscopically, the initial changes consisted of loss of basophilia in the basal portion of the acinar cells, as well as swelling of the zymogen granules with increased secretory activity. Additional changes were vacuolization of the cytoplasm and gradual disappearance of zymogen granules (Figs. 2A and 2B). These vacuoles could not be stained for fat or for glycogen. Eventually the degenerative process progressed to necrosis of the cells. The normal architecture of the acini was completely lost. Many acini underwent ductular transformation (Fig. 2C). Proliferation of ductular cells with active mitosis was observed. In

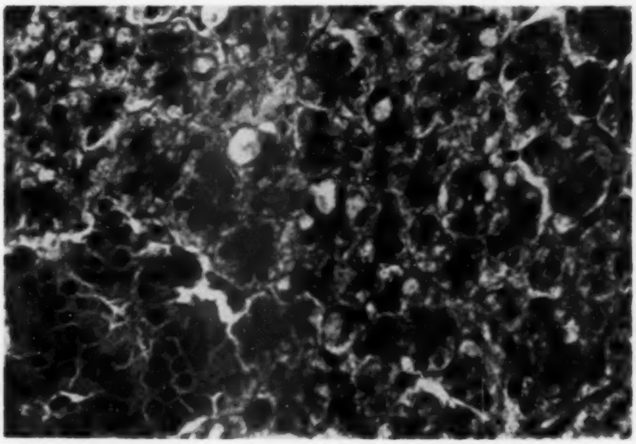

Fig. 2B. — Pancreas of Rabbit 537, given daily intraperitoneal injections of 500 mg. of ethionine for seven days. Note extensive vacuolation of the acinar cells of the pancreas and increased secretory activity (dark areas in center of acini). The islet cells are not damaged (left lower corner). Hematoxylin and eosin; enlarged four times from mag. × 212.

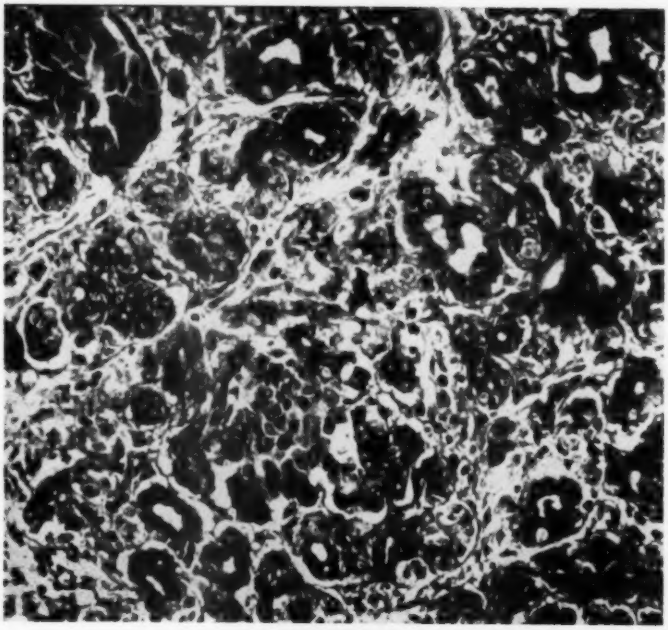

Fig. 2C.—Pancreas of the same animal as Figure 2B. Ductal transformation of the acini in another area of the pancreas. Note regeneration of ductular cells in the interstitial connective tissue. Hematoxylin and eosin; × 250.

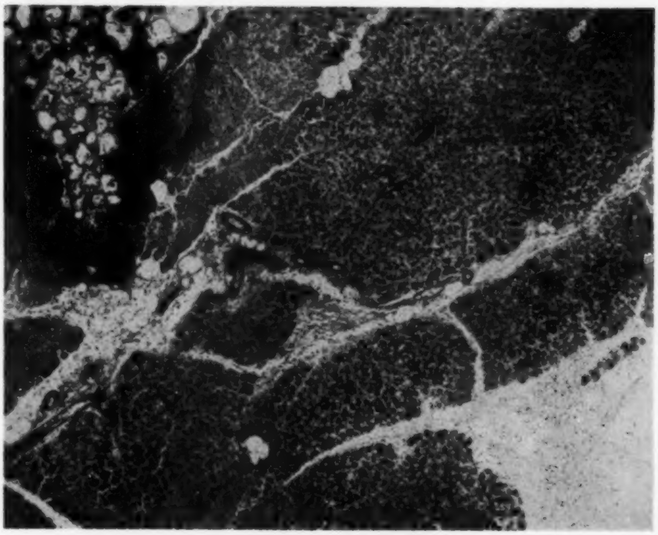

Fig. 2D.—Pancreas of Rabbit 540, given daily intraperitoneal injections of 300 mg. of ethionine for 10 days. Note extensive acute necrosis of the acinar cells and fat necrosis. Hematoxylin and eosin; reduced 15% from mag. × 48.

the interstitial tissue there were edema and inflammatory reaction, with infiltration of polymorphonuclear leukocytes, small round cells, and fibroblasts. Microscopic fat necrosis was seen in six rabbits (Fig. 2D), two of which exhibited marked cellular infiltration, calcification, and foreign-body giant cells. The degree of fat necrosis could not be correlated with changes in serum lipids described in the preceding paper.[1] The elevation of serum amylase appeared to be correlated with the extent of necrosis of the acinar cells.

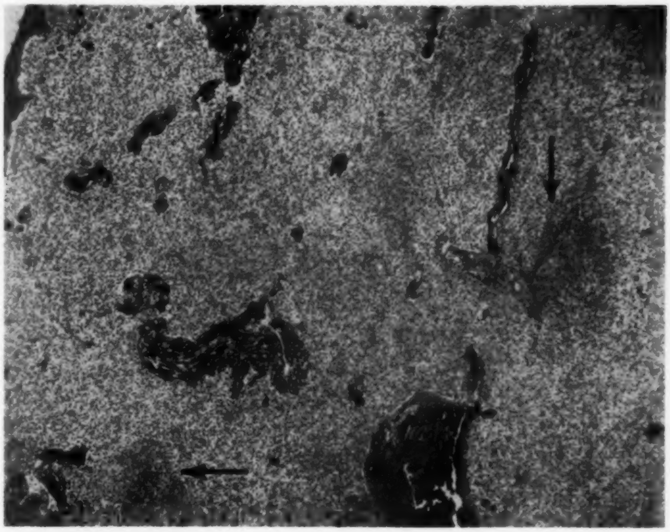

Fig. 3.—Spleen of Dog 611, given ethionine, 100 mg. per kilogram, intraperitoneally every other day for 13 days. Note marked depletion of lymphoid follicles (arrows). Hematoxylin and eosin; reduced 15% from mag. × 48.

The sequence of pathological changes of the acinar tissue in the rabbit was that of an initial stage of stimulation of activity followed by degeneration and destruction of the acinar cells with secondary inflammatory reaction. This observation correlates well with the findings of Castrini in ethionine-treated rats, namely, an initial increase followed by a decrease of ribonucleic acid (RNA) in pancreatic acinar cells.[2]

The pancreas of the dogs exhibited less pronounced gross changes than that of the rabbits. Only in two of the five dogs did the pancreas appear whitish and firm. A few small hemorrhages were seen over the body of the pancreas in one of the animals. This dog also exhibited, in addition, hemorrhages in the nose, oral cavity, stomach, duodenum, and lungs. Microscopic fat necrosis was observed in all six dogs. The focal character of the degenerative and inflammatory changes in the pancreas was more marked in the dogs than in the rabbits. Patches of extensive necrosis of the acinar cells with fat necrosis and infiltration of inflammatory cells were found adjacent to lobules with well-preserved acinar architecture.

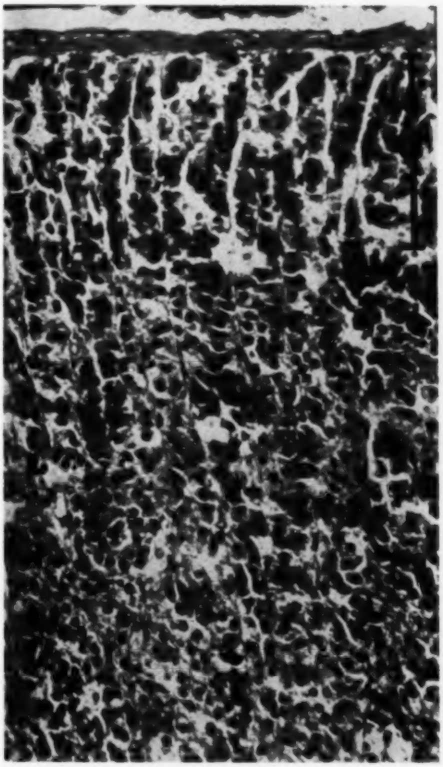

Fig. 4A.—Adrenal of an untreated animal. The thickness of zona glomerulosa is denoted by the vertical line. Hematoxylin and eosin; × 250.

The islet cells were generally well preserved in both the rabbits and the dogs. Only in areas of most extensive tissue de-

struction was identification of the islet cells impossible.

Gastrointestinal Tract.—Gastrointestinal bleeding was more often seen in dogs than in rabbits. Gross hemorrhage was observed in the nose, the oral cavity, the stomach, the duodenum, and the large bowel. One of the dogs died of perforation of the duodenum, with massive hemorrhage into the peritoneal cavity. Gastrointestinal bleeding was seen in only 2 of 19 rabbits. Focal hemorrhages and necrosis of a segment of the small bowel occurred in one rabbit, and extensive hemorrhage into the cecum, in another.

Selective destruction of the chief cells of the stomach was observed by Loring and Hartley in ethionine-treated rats.[3] Such changes were not seen in our material, which, however, was limited to sections from those animals exhibiting hemorrhagic lesions in the digestive tract.

Spleen.—All the spleens appeared dark and softened, as seen in acute toxic conditions. Focal hemorrhages were occasionally noted. Microscopically, there was a striking reduction of Malpighian follicles, especially in the dogs; in two of these, practically no lymphoid tissue could be found (Fig. 3). The red pulp was congested and contained increased amounts of hemosiderin and large numbers of macrophages, suggesting increased red cell destruction.

Adrenal Cortex.—As stated previously, the adrenal glands were enlarged in the majority of rabbits which received ethionine for longer than six days.[1] These animals exhibited elevation of serum lipids. Microscopically, the cortex was markedly thickened. The increase in thickness of zona glomerulosa amounted to two to three times normal (Figs. 4A and 4B). In both the zona glomerulosa and the zona fasciculata the cells were enlarged and vacuolated. In many instances the cells in the latter zone resembled foam cells. In frozen sections these cells were loaded with fat. The amount of fat in the adrenal cortex as judged by Sudan staining seemed to be correlated with the concentration of serum lipids. Increased

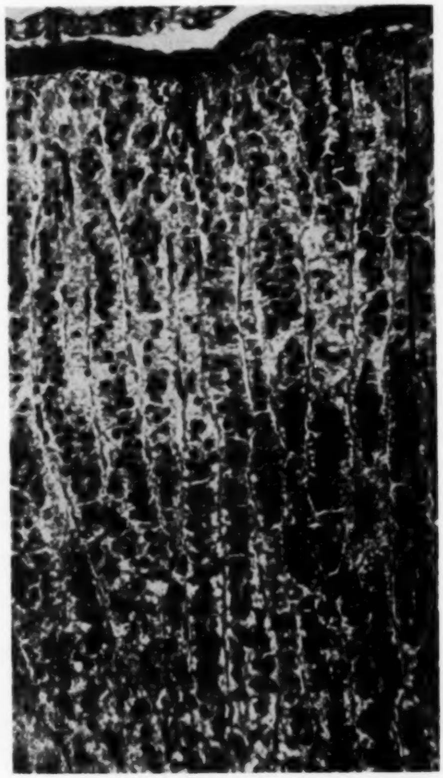

Fig. 4B.—Adrenal of Rabbit 541, after administration of 300 mg. ethionine intraperitoneally daily for 10 days. Note marked thickening of zona glomerulosa and vacuolization of the cells in both zona glomerulosa and zona fasciculata. These vacuolated cells were loaded with fat in frozen sections. Hematoxylin and eosin; $\times$ 250. Serum lipid fractions were also markedly increased in this animal. Cholesterol, total, 296 mg/100 cc.; esterified, 194 mg.; phospholipids, 326 mg., and total lipids, 1220 mg. (Normal serum lipid fractions of rabbit are as follows: cholesterol, total, 50 mg/100 cc.; esterified, 35 mg.; phospholipids, 105 mg., and total lipids, 350 mg.)

amount of fat in the adrenal cortex was found in animals which had elevated serum lipid levels (legend to Fig. 4B). There was absence of hypertrophy, and a reduction of adrenal cortical fat was seen in animals with low serum lipids.

In the dogs gross enlargement of the adrenals was not striking; hemorrhagic areas in the cortex and medulla were observed in two animals. Microscopically, the cells both in the zona glomerulosa and the fasciculata were vacuolated.

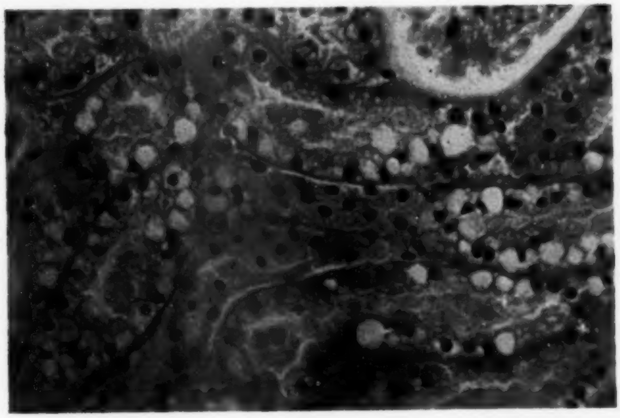

Fig. 5.—Kidney of Dog 612, after administration of 100 mg. ethionine per kilogram of body weight every other day for 16 days intraperitoneally. Note fine and large vacuolation in the tubular epithelium. Hematoxylin and eosin enlarged four times from mag. × 212.

Kidneys.—An amorphous precipitate was seen in Bowman's capsules in most of the animals. In 6 of the 15 male rabbits a peculiar fine vacuolation was found in the proximal convoluted and collecting tubules of the kidneys. In the dogs the vacuolation of the convoluted tubules was more marked (Fig. 5). Frozen section revealed a large amount of fat in the epithelium of these tubules. A few animals showed hyaline and bile casts in the collecting tubules.

Heart and Lungs.—In the myocardium the changes varied from focal fatty degeneration to fragmentation and necrosis. Gross and microscopic hemorrhages in the lungs were observed in some rabbits and dogs.

Pathological Changes in Female Rabbits

This series included four female rabbits, which received 200 mg. of ethionine intraperitoneally per day. Two of these died after four days, and the other two, after seven days. These four rabbits exhibited extensive destruction of the acinar cells of the pancreas. Microscopically, fat necrosis was marked. Fat infiltration of the liver was extensive. Early ductular proliferation of the liver cells was seen in two. The adrenals were enlarged. Extensive hemorrhage in the cecum was also noted in one animal.

The high mortality of the female animals suggested that female rabbits were more susceptible to the administration of ethionine than males. The same sex difference in susceptibility has been observed in rats.[4-5]

Recovery from Effects of Ethionine Administration

Ethionine was given to two rabbits in amounts of 300 mg. daily intraperitoneally for 10 days. It was then discontinued, and the animals were killed 9 and 18 days later. Regeneration of acinar tissue of the pancreas was observed in both animals. In the first animal, certain areas of the pancreas showed loss of basophilic striation and diminution of eosinophilic granules. In the second animal, none of these changes were seen. Marked proliferation of pancreatic ductules and some interstitial cellular infiltration were the only remaining evidence of a repair process. There was no excessive fat in the liver of either animal. The Malpighian follicles in the spleen were well filled with lymphocytes. The adrenals of the first animal (killed after nine days) were still enlarged and showed the microscopic changes described above, while those of the second animal (killed after 18 days) were of normal size and exhibited microscopically complete return to normal. Serum lipids, proteins, lipoproteins, and glycoproteins of these two animals also showed a tendency to return toward normal levels. Some protein material remained in the Bowman's capsules of the kidneys. These limited observations suggest that the severe toxic effects of ethionine are reversible, at least under the experimental conditions used.

Comment

Pathological changes in various internal organs other than the pancreas have been noted by many investigators employing ethionine for the study of experimental pancreatitis. Fatty metamorphosis of the liver [6-8]; renal damage, especially following protein depletion [9]; destruction of chief cells of the stomach and of the submaxillary glands [3]; hemorrhage and ulceration of the bowel,[8,10] and testicular degeneration [11] have been reported. Hepatic tumors due to prolonged feeding of ethionine have been described.[12-13] Increased urinary nitrogen excretion caused by enhanced protein catabolism has been observed.[14] Ethionine given to pregnant rats produced underdevelopment of the offspring and stillbirths.[15] The wide range of pathological changes indicates that ethionine exerts severe systemic toxic effects in the experimental animals.

One would expect that ethionine, a powerful metabolic competitor of methionine, should damage those organs with the most active methionine metabolism—mainly the liver, the small intestine, and the pancreas.[16] Apparently, the primary interference with methionine metabolism leads to a defect in protein synthesis. Earliest pathological findings in the organs observed in this study were varying degrees of degenerative changes followed by necrosis of cells. Inflammation, if present, was usually secondary to necrosis of parenchymal cells. With the use of large doses and after prolonged administration of ethionine, severe toxic changes were encountered in almost all organs.

It is of interest to correlate the pathological findings produced by ethionine with the clinical biochemical observations presented previously.[1] The interference with protein synthesis resulted in rapid weight loss and emaciation of the animals. Progressive diminution of circulating proteins, lipoproteins, and glycoproteins was noted. There was especially marked reduction of serum γ-globulin, probably a result of extensive suppression of lymphoid tissue.[17]

Previous investigators failed to find specific changes in the adrenal glands in rats and in dogs after ethionine administration.[9,12] However, hemorrhage in adrenal glands and decreased fat content were noted in rats.[7-8] In this study most of the animals which received ethionine for longer than six days showed hypertrophy of the adrenal cortex concomitant with a transient elevation of serum lipids. These changes in serum lipids were perhaps in causal relationship with the hypertrophy of the adrenal cortex.[18] On the other hand, the eventual exhaustion of the adrenal cortex may have been responsible for the gradual decrease of circulating lipids, progressing to almost total disappearance. DiLuzio et al. have found marked reduction of circulating lipids in dogs following bilateral total adrenolectomy.[19] On the basis of studies of frozen section stained with Sudan IV, the fat content of the adrenal cortex was increased in some animals and reduced in others. These changes were parallel with the elevation or reduction of serum lipids.

The fatty infiltration of the liver, at least in the rabbits, seemed to be independent of the pancreatic damage in that marked generalized fat infiltration of the liver was often observed in the presence of only mild or moderate damage to the pancreas. It has been shown that after oral administration of small doses of ethionine (2.5 mg. per kilogram) to dogs for a prolonged period of time (five and one-half months) pancreatic atrophy occurred while the liver was normal.[20] Fatty liver has been noted to develop before the depression of pancreatic secretion in rats.[8] All these observations indicate that fat infiltration of the liver produced by ethionine was not causally related to the pancreatic damage.

It is interesting that in the rabbit early ductular-cell transformation in the liver occurred after relatively short periods of ethionine administration. More advanced changes of the same type have been described in rats by Popper et al.[21] after prolonged ethionine feeding. The frequency of

parasitic infestation in the liver of rabbits causing cholangitis and pericholangitis with fibrosis makes the evaluation of any changes in the liver of ethionine-treated animals difficult.

The coagulation defect, previously reported,[1] resulted in macroscopic or microscopic hemorrhages in many organs: the gastrointestinal tract, pancreas, spleen, liver, lungs, and adrenals. It should be emphasized that two factors contributed to the genesis of "hemorrhagic pancreatitis" after ethionine administration. Hemorrhage into the pancreas was a part of the general hemorrhagic diathesis produced by this substance. The degeneration and necrosis of the acinar cells was a part of its generalized systemic toxic effect.

Summary

Ethionine produces extensive damage to various internal organs in the rabbit and in the dog. The pathologic changes consist essentially of degeneration followed by necrosis of parenchymal cells. Inflammation is secondary to tissue destruction. Severe coagulation defects lead to macroscopic and microscopic hemorrhage in various organs.

The changes in the internal organs are often a combination of degenerative changes of the parenchyma with hemorrhagic manifestations. Thus the "hemorrhagic pancreatitis" produced by ethionine is the resultant of the hemorrhagic diathesis and severe tissue damage.

Stimulation of adrenal cortex is observed in the experimental animals. These anatomic changes correlate well with elevation of serum lipids. In the absence of adrenocortical hypertrophy low serum lipid values are encountered.

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Primary Thoracic Myelolipoma

Case Report

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This is the second report on record of a primary thoracic myelolipoma. A retropleural mass of bone marrow, nestled against the right sides of the 9th and 10th thoracic vertebral bodies, was found incidentally at autopsy.

Clinical History

An 80-year-old white man, admitted comatose, had enjoyed good health until the onset of drowsiness five days before. He was afebrile but dehydrated, acidotic, and with a chiefly polymorphonuclear leukocytosis of 16,100. There were lessened breath sounds over the right lower lung field. Despite intensive antibiotic, fluid, and electrolytic therapy, he died, unimproved, 11 hours after admission.

Autopsy gave gross, microscopical, and cultural proof of a fatal Aerobacter aerogenes pneumonia, with secondary acute and chronic purulent pericarditis. General arteriosclerosis and other stigmata of old age existed. Sternal, costal, and vertebral bone marrow were all unremarkable, and no tissue or organ gave evidence of extramedullary hematopoiesis or of any blood disease.

There was a dumbbell-shaped mass, 4 cm. long and 1 cm. in greatest width, nestled against the right lateral body surfaces of the 9th and 10th thoracic vertebrae. The bodies bore matching shallow impressions, but the intervening disk was not at all affected. There was no connection between the vertebral marrow and the mass; the cortical bone between was quite unremarkable. The mass lay behind parietal pleura, in loose areolar endothoracic fascia, was attached nowhere save very slightly to spinal periosteum, and was shelled out with ease. The consistency was springy, the cut surface, uniformly red-tan.

Microscopical examination showed a thick fibrous capsule bearing hemosiderin deposits and a few inflammatory round cells. The inside consisted of hematopoietic and adipose tissue, chiefly the former. The blood-forming elements were in their usual proportions and disposed in a characteristic manner, so that, save for absence of bony spicules, the resemblance to healthy bone marrow was exact (Figs. 1 and 2).

Comment

The encapsulation and bony depression argue long existence; the course and histological picture, benignity. The site where, primitively, dorsal, intermediate, and lateral mesoderm branched is embryologically analogous to retroperitoneum, which is known to have a hematopoietic potentiality in the

Fig. 1.—The general pattern.

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From the Pathological Laboratory, St. Luke's Hospital.

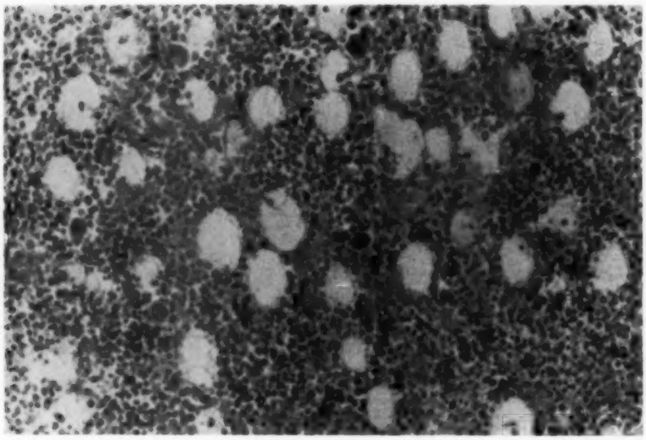

Fig. 2.—The cellular composition.

face of infection.[1] Adipose and areolar tissues have the capacity to undergo lymphadenoid transformation under stress,[2] and there is record of blood formation developing in a recurrent mesenteric lipoma.[3] Instances of hematopoietic cells in such sites as the rectus abdominis [4] and sciatic epineurium [5] are described. Although nobody knows whether such changes come from activated primitive cell-rests or, as some think, by heteroplasia,[6] there is little doubt that they do occur.

Bone marrow arises from splanchnic and mesodermal derivatives—ordinarily, from hemocytoblasts, and unusually, from mesenchyme. Bone marrow masses may exist heterotopically. Some can be linked with known anemias and may be called secondary; others, occurring without any conceivable stimulus to extramedullary hematopoiesis, may be considered primary.[7] How the latter arise is obscure. Some are of long duration—perhaps congenital; others, to judge by clinical histories, begin in adulthood.

Lacking etiological and pathogenetic facts, one may best characterize a lesion by description. The component tissues and occurrence as demarcated masses prompt the term myelolipoma. The absence or presence of known hematopoietic stimuli determine the qualification as primary or secondary. Location completes the name.

The adrenal masses commonly called myelolipoma fit comfortably into this system.

Excepting adrenal glands,[8-10] primary myelolipomata are rare: two have been described in presacral tissue [11,12]; one, in the retroperitoneum,[13] and a fourth, in the thorax—bilaterally beneath subcostal pleura.[14]

Of secondary thoracic myelolipoma, four instances are recorded—all paravertebral or subpleural.[15-18]

A malignant counterpart may exist: in one case [19] there was a retroperitoneal mass, histologically indistinguishable from myelogenous leukemia but without leukemic involvement of blood or bone marrow; in another,[20] an orderly but rapidly fatal progression of adipose, myeloid, and lymphoid cells throughout abdominal adipose tissue.

In our case there was no hemoglobin determination, but the previous history, together with the short-lasting fatal infection and postmortem findings, justly exclude any anemia or known hematopoietic stimulus and permit designation of this myelolipoma as primary.

Summary

A primary thoracic myelolipoma, the second on record, is reported.

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Selective Toxicity of Radioactive Sulfate for Mouse Cartilage and Bone Marrow

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Tracer amounts of radioactive sulfur (S^{35}) given as sulfate are selectively fixed and retained for extensive periods in normal human [1] and animal [2] cartilage and in malignant tumors of human cartilage.[1] Sulfate S^{35} is also taken up by mucin-secreting epithelia [3] and by some mucin-secreting carcinomas.[4] This fixation of S^{35} in cartilage and mucinous epithelium is apparently due to its incorporation into the sulfated mucopolysaccharides.

Since these tissue affinities suggest a therapeutic value, it seemed desirable to determine whether S^{35} sulfate can produce tissue damage and, if so, at what dose level. The amounts of carrier-free S^{35} that can be given are not limited by its chemical toxicity, as sulfate is a common ion of body fluids. Although the radioisotopes presently used for therapy as internal emitters have stronger radiations than has S^{35}, the relative weakness of the radiation of S^{35} (average 55 kev) does not preclude toxic radiobiological effects. Its energy is greatly in excess of that needed for the formation of ion pairs (0.032 kev).[5] Radiotoxic deaths have been produced in mice by administration of as weak a β-emitter as tritium.[6]

In the series of experiments that will be described, progressively larger amounts of S^{35} sulfate were injected into mice. The highest doses produced early death by bone marrow destruction and leukopenia; smaller doses selectively destroyed growing cartilage cells, and the animals survived.

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From the Radioisotope and General Medical Research Units, Veterans' Administration Center, Martinsburg, W. Va. (Dr. Gottschalk and Dr. Beers) and the Department of Pathology, George Washington University School of Medicine, Washington, D. C. (Dr. Gottschalk).

Materials and Methods

Young adult male B alb/C mice (obtained from Jackson Memorial Laboratory) were used in four separate tests (Table 1). All animals received Purina Checkers and water ad lib. The mice given injections and the control mice were kept in cages with screen floors raised 1 to 2 in. In the weeks following injection the droppings were removed frequently in order to decrease the external irradiation from the isotope in the urine and feces. All animals were weighed at regular intervals. In Tests III and IV the mice were housed in individual cages and the food and water consumed (including the wasted portions) were measured every few days. Numerous blood samples were obtained from the tail before injection and for at least one and one-half months after injection. They were used for white and red blood cell counts and differential leukocyte counts. Studies on all mice before injection provided additional control values.

The radioactive sulfur was obtained from the Radioisotope Division of Oak Ridge National Laboratory in the form of carrier-free $H_2S^{35}O_4$ in weak HCl. Neutral isotonic solutions of radioactive sodium sulfate were prepared by adding

Table 1.—*Plan of Injections of Radiosulfate to Mice*

Test	Total Dose S^{35}, Mc./Gm.	Route	Volume Injected,* Ml.	Total Na_2SO_4,† µg.
I	0.001 to 0.1	I. V.	0.2	8
I control	0	I. V.	0.2	8
I control	—	0	0	—
II	0.08	I. P.	0.3	12
III	0.2 to 1	I. P.	3×0.5	? ‡
III control	0	I. P.	3×0.5	0
IV	0.5 to 5	I. P.	0.05 to 0.5	3 to 31
IV control	0	I. P.	0.2 to 0.5	17 and 43

* Calculated for 20 gm. mouse.

† Total weight of carrier and isotopic sulfate given to 20 gm. mouse.

‡ Concentration of sulfate in original isotope solution unknown, no carrier added.

NaOH, NaCl, and pyrogen-free H_2O. Small amounts of Na_2SO_4 were added as carrier in Tests I and II. The doses injected were based on the S^{35} activity and on the body weight of the mice on the day of injection.

In Test I, 7-week-old mice were given small doses of radioactive sodium sulfate as single intravenous injections. The controls were divided into two groups, those receiving no injection and those receiving equivalent doses of saline with trace amounts of nonradioactive sulfate.

In Test II, 16-week-old mice received single doses of S^{35} by the intraperitoneal route.

In Test III, increasing total doses of radiosulfate were administered intraperitoneally to mice 7 weeks old. The isotope was given in three doses at 11-hour intervals. The controls were given injections of saline solution.

In Test IV, larger single doses of S^{35} were administered intraperitoneally to 13-week-old mice. Controls in this series received equivalent amounts of saline and nonradioactive sulfate.

The rate of disappearance of S^{35} from the blood was determined on numerous 10 cu. mm. samples of blood obtained from four mice of Test III in the first three days after the isotope injections. These samples were hemolyzed and dried on planchets. Their radioactivity was measured with a thin-window Geiger-Müller tube and compared with that of the injected solution.

The animals in Tests I and II were killed in groups, 42, 83, and 104 days after injection. The mice of Test III were kept until their natural death, probably from intercurrent infections (average survival: 185 days for the mice given injections of S^{35} and 177 days for the controls). The mice of Test IV were killed after 126 days. Six additional mice, not included in the Tables, received 1 mc. per gram, 0.4 mc. per gram, or the control solution at the same time as the mice of Test IV. They were killed 3½ hours and 19 hours after injection, for study of the early histological changes.

All mice were autopsied. In Tests I and II sections were regularly taken from the liver, spleen, suprarenal gland, kidney, testis, heart, lung and great vessels, skin, sternum, bones adjacent to knee, and vertebrae and cord. They were fixed in formalin and in Bouin's solution. In Tests III and IV the same tissues and, in addition, sections of the pancreas and duodenum, jejunum, large intestine, bones adjacent to elbow, and base of tail were fixed in formalin solution. Carnoy's fluid was used for the mice killed on the day after injection. The bone specimens, with the exception of the sternum, were decalcified. All sections were stained with hematoxylin and eosin.

Results

Lethal Dose and Effects on Blood Cells.—Table 2 summarizes the effects of S^{35} sulfate on the survival and blood cell counts of mice. No radiation death was observed in these tests after doses up to 1 mc. per gram of body weight. Doses of 2 mc. per gram produced death in two out of three mice, and 5 mc. per gram killed, after a shorter delay, the two only mice given injections. These mice had increasingly severe leukopenia. A mouse given an injection of 5 mc. per gram had a leukocyte count of 580 one hour before death. Leukopenia appeared to be the main cause of their death.

After lower doses (0.5 and 1 mc. per gram) only transient leukopenia occurred. The leukocyte counts were depressed on the

TABLE 2.—*Radiotoxic Effects of $Na_2S^{35}O_4$ on Mice*

Test	Dose S^{35}, Mc./Gm.	No. of Mice Injected,	No. of Mice Dying Within 30 Days, Delay After Injection	Average Blood Cell Counts After Injection *		
				Mice Studied, No.	WBC, Thousands	RBC, Millions
I	0.001	4	0	4	8.4	9.7
I	0.01	4	0	4	11.4	9.9
I	0.1	3	0	2	11.2	10.3
I Control	(saline-sulfate)	3	0	3	12.0	10.4
I Control	0	3	0	3	11.6	10.9
II	0.08	2	0	2	13.1	10.9
III	0.2	4	0	1	10.2	9.9
III	0.5	4	1 † (12th day)	1	5.0	9.2
III	1.0	3	0	3	5.9	9.0
III Control	Saline	4	0	1	10.8	9.6
IV	0.5	2	0	2	7.1	9.0
IV	1.0	2	0	2	6.3	8.5
IV	2.0	3	2 (6th, 14th day)	3	2.8	8.6
IV	5.0	2	2 (5th, 5th day)	2	2.1	8.6
IV Control	(saline-sulfate)	5	0	5	17.8	10.2

* Average of all blood cell counts made within 15 days after injection (generally 3 or 4 blood samples per mouse).
† Probably not radiotoxic death.

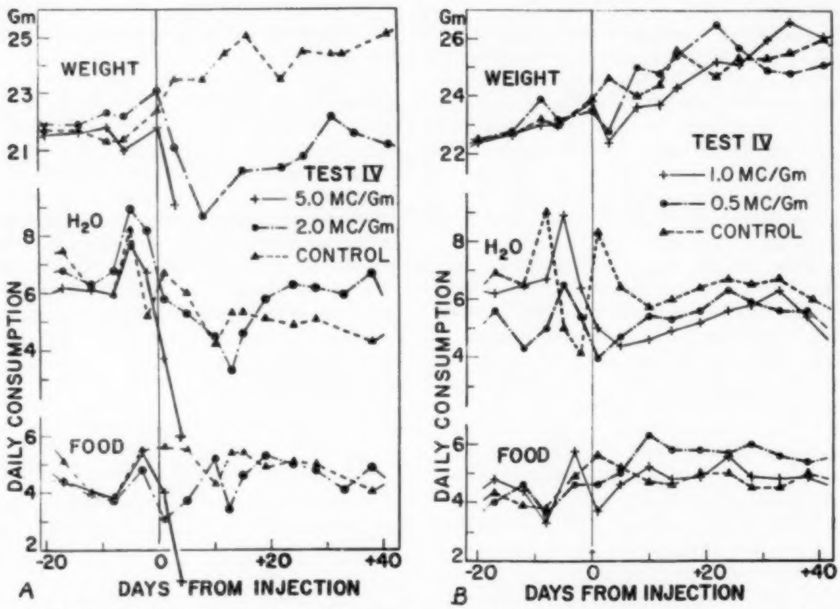

Fig. 1*A* and *B*.—Effects of S^{35} sulfate on mouse weight and food and water consumption (Test IV). The group averages of daily consumption are plotted in the middle of the periods covered. The controls in *A* received 0.025 ml. of saline and sulfate solution per gram body weight, and those in *B*, 0.01 ml. per gram.

day after injection, reached a minimum generally after 5 to 13 days, and returned to values close to the preinjection level after 3 weeks. Both granulocytes and lymphocytes were decreased. The erythrocyte counts were slightly lowered after doses of 1 mc. per gram and up. The hematologic changes are separately reported in greater detail.[7] The low doses of Tests I and II had no effects on the leukocyte and erythrocyte counts.

Food and Water Intake and Growth.—Five millicuries per gram produced a severe decrease of food and water consumption as observed in radiation injury. The food and water intake seemed slightly depressed after 2 mc. per gram (Fig. 1*A*). At lower doses (Fig. 1*B*) and in Test III no such effect could be distinguished from the incidental variations of intake, probably related to handling, room temperature, etc. Diarrhea, possibly indicative of intestinal toxicity, was observed four or five days after injection in the two mice that had received the highest dose and in one mouse of each of the two next groups.

The mice of Test IV were almost fully grown. The two highest doses produced an immediate and marked fall of body weight (Fig. 1*A*), and the two next doses, a slight transient fall (Fig. 1*B*). In the latter mice and in the somewhat younger mice of Test III, which also showed a slight temporary weight loss after 1 and 0.5 mc. per gram (Fig. 2), no permanent impairment of the gain of weight was produced. The doses used in Tests I and II had no effect on the body weight. On a weight basis these doses were 10 to 1000 times higher than the largest amounts of S^{35} sulfate used for tracer studies in humans (about 0.1 mc. per kilogram).[1]

Microscopic Observations.—The histological changes observed in the few mice killed soon after injection were generally similar to those described after external total-body irradiation.[8,9] Three and one-half hours after

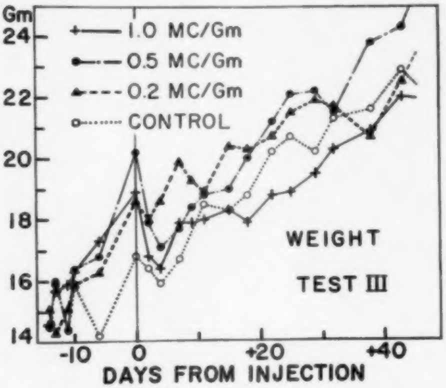

Fig. 2.—Effect of S^{35} sulfate on mouse weight (Test III).

S^{35} injection mitoses were very rare in the crypts of the small and of the large intestine; they returned to normal after nineteen hours. The lumen of the crypts contained a small amount of cellular debris after 3½ and 19 hours. After the latter interval the tips of some villi appeared swollen. In the mice which died within a week after large amounts of S^{35} were given, some crypts of the small intestine were irregularly dilated. The mucus-secreting epithelium of the colon showed degenerative changes only in these mice; the glands had irregular areas of epithelial regeneration, and some cells had unusually large nuclei (Fig. 3). No changes in the intestinal lining were found in mice autopsied at later dates.

On the day after injection the lymphoid follicles of the intestine contained some nuclear debris. These follicles were atrophic, and the spleens were small in the mice dying after maximal doses of S^{35}.

In all the mice dying after injection of 5 or 2 mc. per gram the differentiated hema-

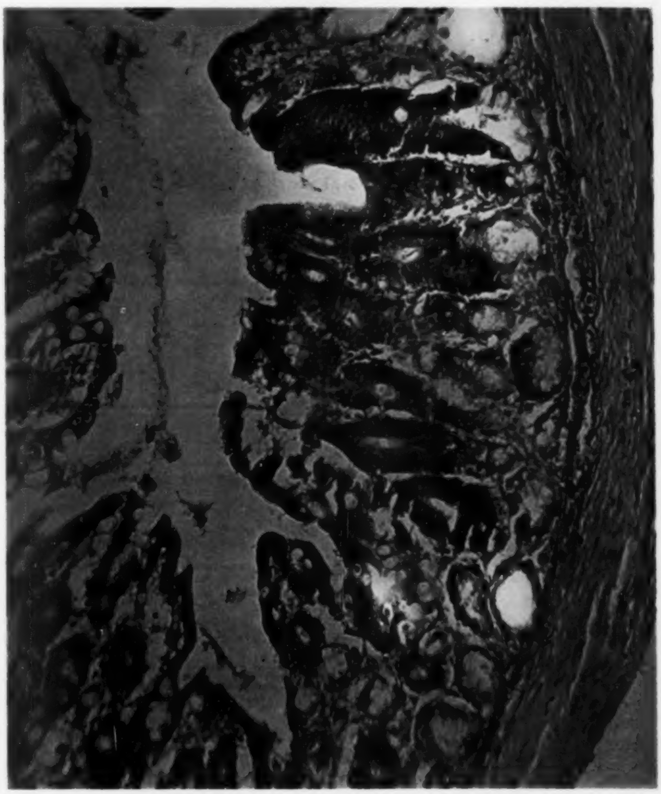

Fig. 3.—Colon of Mouse B 665, Test IV, which died six days after receiving 2 mc. per gram. The glands are irregular, with dilated secreting portions and regenerating areas. Swollen epithelial cells with large nuclei. Hematoxylin and eosin; reduced slightly from mag. × 150.

Fig. 4.—Upper epiphysis of tibia of Mouse B 670, Test IV, control, killed 14 days after sham injection. Orderly columns of cartilage cells in epiphysial plate. Active bone marrow. Hematoxylin and eosin; reduced slightly from mag. × 195.

topoietic cells had almost completely disappeared from the bone marrow; rare reticuloendothelial cells were scattered in lakes of red blood cells (Figs. 5 and 6). These mice also had some interstitial hemorrhage in the lungs and periosteum. After 19 hours the bone marrow was already congested and the number of marrow cells was reduced, with the exception of the megakaryocytes. Beginning regeneration of the bone marrow cells was evident in the mouse that died 14 days after injection of 2 mc. per gram. In all the mice autopsied after longer intervals, either killed or dying of incidental conditions, the bone marrow and lymphoid structures had completely recovered.

Severe degeneration of the epiphysial cartilage was observed in all the mice autopsied one month or more after injection of 0.5 mc. per gram and of higher doses. Characteristically S^{35} destroyed the chondrocytes of the proliferating zone of the epiphysial plates in the vertebrae and in all the long bones studied. The columns of cartilage cells disappeared. Bizarre hypertrophied cells, with dense cytoplasm and pycnotic or fragmented nuclei, and, later, empty lacunae were scattered haphazardly in the ground substance of the markedly broadened plates

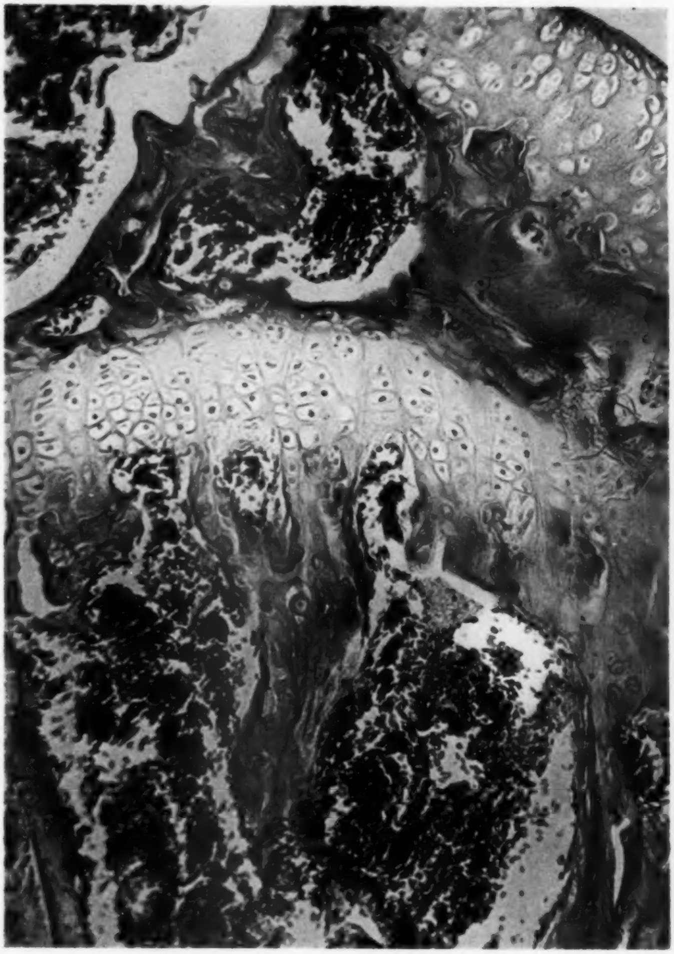

Fig. 5.—Upper epiphysis of tibia of Mouse 662, Test IV, five days after receiving 5 mc. per gram. Epiphysial plate of normal width and architecture, with pycnotic cells. The marrow contains only erythrocytes and endothelial cells. Hematoxylin and eosin; reduced slightly from mag. × 190.

(Figs. 7 and 8). Close to the epiphysis, the bony lamellae were absent or irregular and endochondral bone formation was arrested. Broad and irregular completely necrotic epiphysial plates (Fig. 9) persisted in the shafts up to maximum periods of observation; their abundant irregularly staining ground substance contained empty lacunae and no chondrocytes. In some mice given injections of 0.5 or 1 mc. per gram there was an abortive attempt at formation of a new plate that itself degenerated, while the necrotic primary plate was carried as a sequestrum into the shaft (Fig. 10). These "double" plates were found in the femur and tibia near the knee joint but not in the humerus and vertebrae. After 0.2 mc. per gram the mice had some cartilage degeneration and more successful regeneration (Fig. 11). After only 0.1 mc. per gram some epiphysial plates were slightly disorderly and small islands of degenerated cartilage tagged by deposits of S^{35} were carried down into the shaft. As the radiation of S^{35} has a very short range, the necrotic cartilage plates were adjacent to normal bone marrow late after injection. On the contrary, the mice dying after fatal doses had, side by side, aplastic bone marrow and epiphysial cartilage that showed only a slight decrease in

Fig. 6.—Higher magnification of Figure 5. Hematoxylin and eosin; reduced slightly from mag. × 510.

the number of chondrocytes and some nuclear condensation.

Only minimal cellular changes and no necrosis were observed after varied intervals in the hyaline cartilage of the articular surfaces and of the xiphoid process, probably because of the lower radiosensitivity and slower rate of cellular multiplication of hyaline cartilage.

No other early or late toxic effect of S^{35} could be recognized in the different organs and tissues of the mice autopsied after varied intervals. The testes remained active, but slight changes may have escaped detection because of the complex organization of the seminiferous tubules in mice. There was no morphological evidence of renal damage. Most of the mice that were not killed, both those given injections and controls, died of pneumonia.

One mouse of Test III died of myelogenous leukemia 294 days after injection of 1 mc. per gm.; this condition may have been spontaneous, as leukemia is not infrequent in this strain,[10] or it may have been induced or accelerated by the radiation, as the development of leukemia is promoted by external radiation and also by internally administered radioisotopes [11] (P^{32}, I^{131} etc.). Long-term observations on a larger number

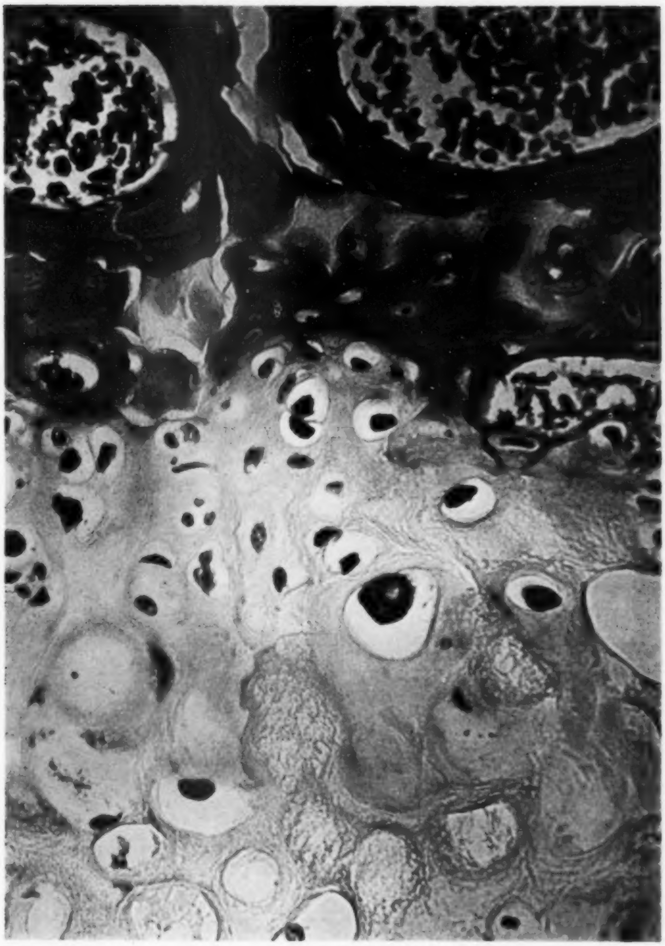

Fig. 7.—Upper epiphysis of tibia of Mouse 625, Test III, 35 days after receiving 0.5 mc. per gram. Broadened disorganized epiphysial plate, with degenerating chondrocytes. Active bone marrow (high magnification). Hematoxylin and eosin; reduced slightly from mag. × 450.

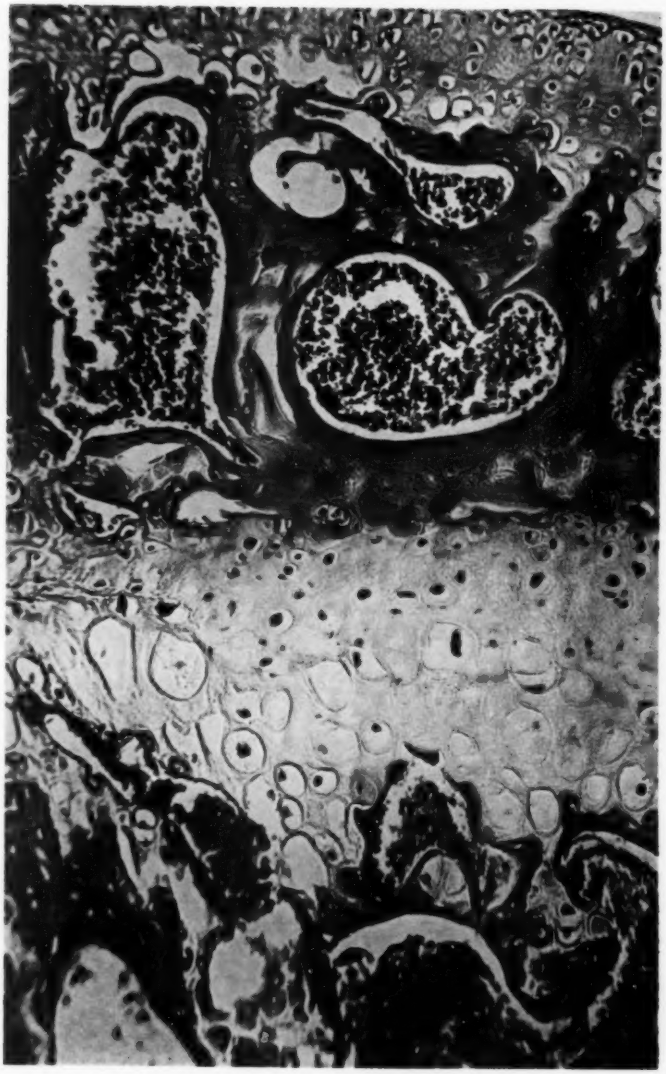

Fig. 8.—Same slide as Figure 7, lower magnification. Hematoxylin and eosin; reduced slightly from mag. × 185.

Fig. 9.—Knee of Mouse 656, Test IV, 126 days after receiving 0.5 mc. per gram. In femur (above) and tibia, broad degenerated epiphysial plates, ossification arrested. Articular cartilage preserved. Active bone marrow. Hematoxylin and eosin; reduced slightly from mag. × 56.

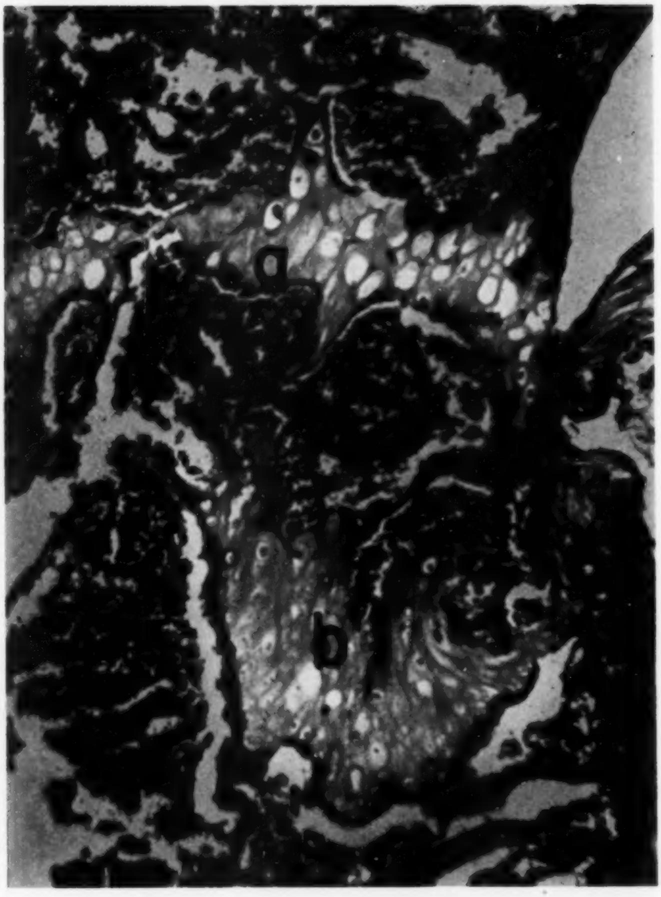

Fig. 10.—Lower epiphysis of femur of Mouse 621, Test III, 172 days after receiving 1 mc. per gram. Degenerated original (*a*) and secondary (*b*) epiphysial plates. The shaft is above. Active bone marrow. Hematoxylin and eosin; reduced 10% from mag. × 93.

Fig. 11.—Upper epiphysis of tibia of Mouse 627, Test III, 254 days after receiving 0.2 mc. per gram. Necrotic rests of original plate in cortex and medullary cavity (*a*) and regenerated epiphysial plate (*b*). Active bone marrow. Hematoxylin and eosin; × 87.

of mice would be desirable to ascertain whether large doses of S^{35} promote the development of leukemia.

Comment

The selective destruction of epiphysial cartilage cells is explained by the high uptake of radiosulfate in cartilage and by its long retention in its ground substance,[1,2] so that the radiotoxic effects appeared after some delay in cartilage and were maintained up to the term of the experiments. On the contrary, S^{35} disappears rapidly from the blood and from most tissues of the rat[12] and of man.[13] In the bone marrow the uptake is relatively high,[1,12] and the turnover is slower than in most tissues, although faster than in cartilage.[12] This explains the early irreversible injury to the radiosensitive marrow cells produced by the largest doses of S^{35}. Radiosensitivity also explains the early changes in lymphoid tissue and intestinal epithelium. The transient high concentration of S^{35} in mucus may contribute to the latter effect.

The short range of the radiation of S^{35} permits highly specific injury to the epiphysial cartilage after nonfatal doses. The histological changes are different from those produced by "bone seekers," like radiostrontium, that damage the bone of the metaphysial spongiosa and produce chronic changes in the bone marrow after much lower doses.[14]

It was not the purpose of these tests to establish accurately the 50% lethal dose for mice of S^{35} sulfate. The results, however, indicate that the doses fatal for these mice were above 1 mc. per gram. In tests concurrently conducted at Oak Ridge National Laboratory, doses of radiosulfate up to 32 mc. had no toxic effects on adult rats.[15] Odeblad and Boström [16] failed to damage the genital organs and to impair the fertility of female mice injected with 0.06 mc. per gram $Na_2S^{35}O_4$. These high values merely reflect the weakness of the radiation and the rapid turnover of S^{35} sulfate in most tissues. In rats 67% of the dose is excreted in the urine in the first 24 hours,[12] and in man, 55% to 78%.[13] In man the blood concentration of S^{35} reaches 10% of the average dose injected per gram of body weight after 24 to 50 hours.[13] These results indicate an effective biological half-life of approximately 10 hours for the entire body during the first days after injection in man and in the rat.

Direct measurements of the radioactivity of the blood made in the mice of Test III indicated that the turnover of S^{35} was still faster in mice. The blood concentration reached 10% of the average injected dose 10 hours or less after injection. This would indicate an effective biological half-life of approximately 2.5 hours in the blood and probably in the whole mouse. Using this half-life value and disregarding the small fractions having a slow turnover, we can calculate that 2 mc. per gram would deliver a cumulative total-body irradiation dosage of 990 rep.* The observed lethal doses of S^{35} sulfate (2 mc. per gram and up) † are also in the expected range when compared with the published midlethal doses for mice of other radioisotopes (H^3, P^{32}, and Na^{24}),[6,14] if their respective effective energies and assumed effective half-lives are taken into consideration.[6,18]

The destructive effect of intermediary doses of S^{35} sulfate on the epiphysial cartilage seems at variance with the lack of impairment of increase of body weight. These mice had, however, completed most of their growth when S^{35} was injected. In more recent experiments the injection of S^{35} sulfate into rats at the age of 2 weeks completely arrested the increase in length of the long bones and produced achondroplastic-like dwarfs.[7] Lower doses were required than in the mouse, probably because of the slower turnover of S^{35} in the rat and of its higher uptake in the cartilage of young animals.[19]

The present experiments show that because of metabolic specificity doses of S^{35} sulfate under the lethal level produce irreversible damage to the cartilage of the epiphysial plates. The concentration of radiosulfate in chondrosarcomas generally exceeds that in adult cartilage, and S^{35} localizes mainly in the growing portions of these tumors.[1,13] There is thus some rationale for attempts of therapy with radiosulfate in cases of widespread chondrosarcomas that have a hopeless prognosis and show a favorable differential uptake by tracer studies. The remote hope of interfering with the progress of these tumors is obviously tempered by the difficulty of obtaining sufficient differential concentration of the radioisotope in all neoplastic foci and by the problems of radioresistance. Any therapeutic attempt in man would also be extremely expensive because of the large amounts of S^{35} sulfate needed to produce radiotoxic effects. Additional studies may be desirable before the

* Formula of Evans.[17]

† In recent tests conducted in this laboratory the toxic doses were lower: 1 mc. per gram was fatal for tumor-bearing mice, and 0.5 and 0.2 mc. per gram regularly produced severe cartilage degeneration.

observations in the mouse are translated into attempts at human therapeutic application.

Summary

Doses of 0.001 to 5 mc. per gram S^{35} sulfate were injected into young adult mice. The effects on body weight, food and water intake, blood cells, and varied tissues were studied. The highest doses produce death from destruction of the hematopoietic tissues. After 0.5 and 1 mc. per gram the epiphysial cartilage plates are selectively and irreversibly destroyed and only temporary leukopenia occurs. These toxic effects are related to the metabolic affinities of S^{35} sulfate. They might warrant attempts at therapy with S^{35} sulfate in cases of terminal widespread chondrosarcomas.

Mrs. P. O. Miller and Mr. E. R. Ridgeway gave technical assistance; Mr. L. Eller supplied the histological preparations, and Mr. H. Busey supplied the photographs.

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Effects of Beta-Aminopropionitrile upon Wound Healing

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The work of Geiger et al.[1] and Ponseti[2] demonstrated that the seeds of Lathyrus odoratus have pronounced effects upon certain connective tissues, including particularly bone, cartilage, and aortic wall. Further investigations identified the toxic substance in these seeds to be β-aminopropionitrile.[2-4]

Several studies have been reported suggesting that the activity of β-aminopropionitrile may be related to the growth and proliferation of connective tissue rather than to its maintenance[1,2] and also to concomitant mechanical stress.[5,6] Since wound healing is characterized by the formation of new fibrous tissue and since this takes place in the absence of any significant mechanical stress, it was thought that wound healing might serve as an experimental model for more accurate determination of the effect of β-aminopropionitrile on the formation of fibrous connective tissue.

Materials and Methods

Male rats of the Wistar strain, 21 to 23 days old at the beginning of the experiment, were divided into four groups of eight rats each; two of these groups served as controls, and two, as experimentals. Similarly, adult male rats of the Wistar strain, weighing 284 to 440 gm. at the beginning of the experiment, were divided into four similar groups of eight rats each.

All animals were fed Purina Fox Chow; 0.4% β-aminopropionitrile fumarate * was added to the diet of the experimental animals. In order to avoid the effects which variations in food intake might have, the rats were pair-fed. The members of each pair were selected so that their weights were as nearly the same as possible. Each rat was caged individually.

On the ninth day after the animals were started on their diets, a wound was made in the right posterolateral chest wall close to the vertebral column. This area was selected because it is inaccessible to the mouth and paws of the animal and is unaffected by the hernias which frequently develop in the posterolateral abdominal wall as a result of the administration of the drug. The hair was clipped with an electric clipper. The wounds were made with use of pentobarbital (Nembutal) anesthesia, without aseptic technique. A circle was stamped on the unstretched skin of the rats by means of a circular punch of 1 cm. diameter which had been previously inked by touching on a fingerprint pad. The skin contained within the circle and the underlying panniculus carnosus were then excised with curved scissors. Care was taken to make the incisions perpendicular to the surface. All wounds were left to heal without any protective dressing, and in no case were the crusts purposely removed.

All rats were inspected every second day and weighed every third day. Pictures of the wounds were taken on the 3d, 6th, 9th, and 12th day, with use of a 35 mm. Exakta camera. The wound-camera lens distance was kept constant, and a centimeter ruler was always photographed with the wound. This technique allowed accurate comparison of the size of the wound as represented on the prints. The wound contraction was quantitated by measuring on the prints with a planimeter the area outlined by the original edges of the wounds. The original inked circle soon disappeared, and the line at which hair growth ceased was subsequently used for the identification of the original wound edge.

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* The β-aminopropionitrile fumarate was supplied by Dr. Robert D. Coghill, Director of Research, Abbott Laboratories, North Chicago, Ill.

One control and one experimental group of weanling and adult rats were killed on the third day after wounding. Similar groups of adult rats were killed on the 12th day after wounding. The high mortality in the weanling rats made it necessary to kill them on the 9th instead of the 12th day. Since only three experimental weanling rats were alive on the ninth postwounding day, this latter part of the experiment was repeated, with use of 12 rats per group. Four experimental rats survived for nine days in this repeat experiment, and so a total of seven experimental and seven pair-fed control weanling rats were available for the study of nine-day-old wounds.

All rats were killed with ether. The wounds with their surrounding tissues were excised, fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Observations

General.—All rats developed changes characteristic of lathyrism. In the weanling rats the peculiar gait and mandibular exostoses were present on the sixth experimental day. Well-developed deformities of the sternum appeared on or after the eighth experimental day, while hernias were first observed two days later. In none of the weanling rats were aortic aneurysms observed. In the adult rats, mandibular exostoses developed on the 10th to 12th experimental days, but peculiar gait, sternal deformities, hernia, and aortic aneurysms were not observed.

In the weanling rats, there was no difference in weight gain between the experimental animals and their pair-fed controls during either the 12 or the 18 days on the diet. In contrast, there was a great difference in weight gain between the above two groups and a group of rats of the same strain, sex, and age which was fed ad libitum for use in another experiment (Fig. 1). Similarly, the weights of the adult experimental animals were not different than those of their pair-fed controls.

Gross Observations of the Wounds.—The most striking gross difference between the wounds of the experimental and control weanling rats was in their rate of contraction (Fig. 2). The percentage decrease in initial wound area on the third, sixth, and ninth days after production of the wound was computed by dividing the decrease in wound area in square millimeters by the wound area on zero day and multiplying the quotient by 100. The results are graphically represented in Figure 3. The mean percentage decrease in intial wound area on the third, sixth, and ninth days is 49±5, 76±4, and 83±5, respectively, in the control rats, and 26±, 34±4, and 43±5, respectively, in the experimental rats. Thus, in the experimental animals the total wound contraction in each period of observation was considerably smaller than that of the controls. It is worth while mentioning that in the experimental rats the mean percentage decrease in initial wound area on the ninth day was smaller than that of the control rats on the third day.

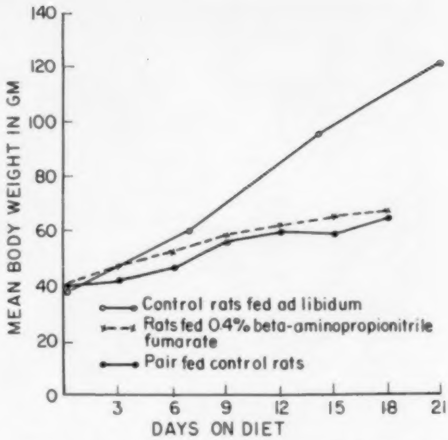


Fig. 1.—Weight curves of weanling rats fed 0.4% β-aminopropionitrile fumarate as compared to pair-fed and ad libidum-fed controls.

The above observations indicated that β-aminopropionitrile inhibits wound contraction. It was then decided to determine whether the inhibitory effect is of the same degree throughout the experimental period or more pronounced during certain stages of wound healing. For this purpose the per cent decrease in wound area during each three-day period was determined. The wound area at the beginning of each period was considered as 100%, and the percentage

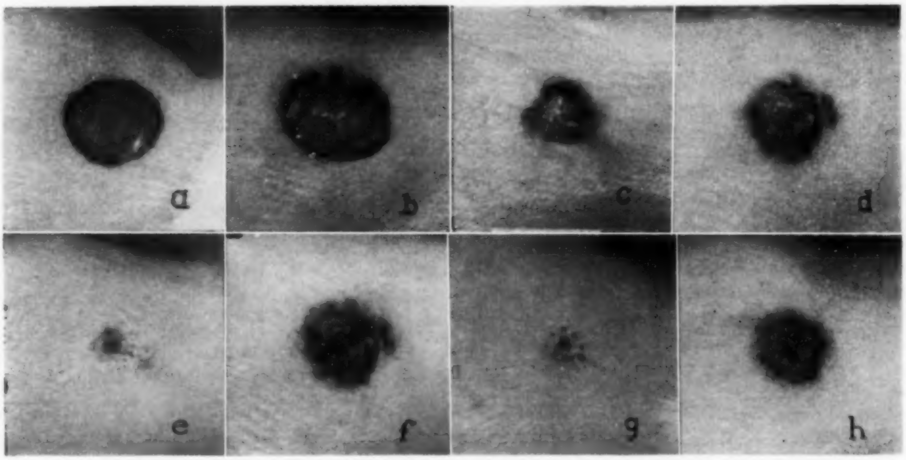


Fig. 2.—Photograph demonstrating the difference in size of wound areas between a weanling rat receiving 0.4% β-aminopropionitrile fumarate and its pair-fed control. (*a*) and (*b*) Zero day. (*c*) and (*d*) Three days. (*e*) and (*f*) Six days. (*g*) and (*h*) Nine days. (*a*), (*c*), (*e*), (*g*) Control. (*b*), (*d*), (*f*), (*h*) Experimental.

decrease was computed by dividing the decrease in wound area during the period by the size of the wound at the beginning of the period and multiplying the quotient by 100. The mean percentage decrease in wound area during the zero- to three-day, three- to six-day, and six- to nine-day periods was 49±5, 54±5, and 27±5, respectively, for the controls and 26±5, 10±5, and 14±5, respectively, for the experimental animals. It can be seen that the amount of wound contraction occurring during the first two periods is much greater in the control than in the experimental rats. It may be noteworthy to mention that this difference between the control and experimental animals is much more pronounced in the three- to six-day period than in the zero- to three-day period. The amount of contraction occurring in the third period is only slightly greater in the control rats.

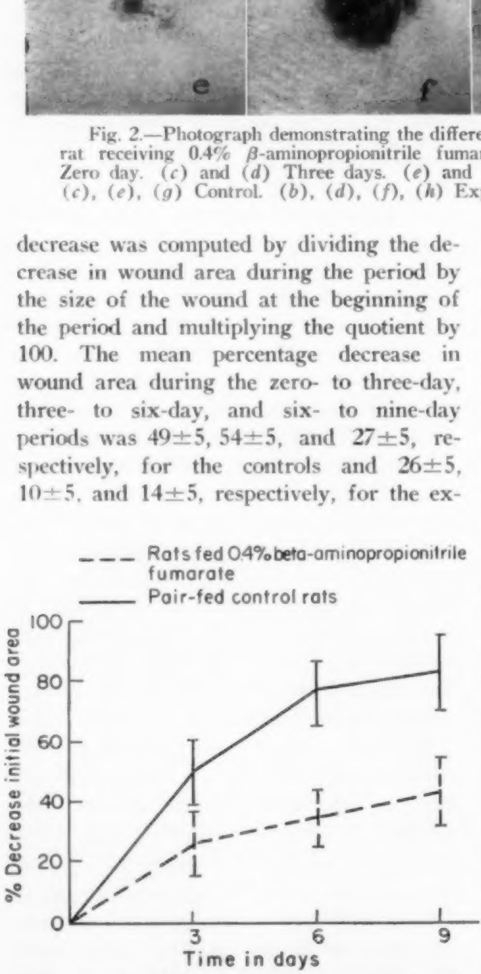


Fig. 3.—Graph demonstrating the mean per cent decrease in initial wound area of weanling rats receiving 0.4% β-aminopropionitrile fumarate and their pair-fed controls.

During the first few days after wounding, some firmness, swelling, and redness developed in the rim of skin surrounding the wound of the control rats. This did not occur in the experimental animals. In the rats of the latter group the crust was consistently darker in color and more elevated than that of the controls.

The healing of the wounds of the adult experimental animals did not differ on gross examination from that of their pair-fed controls.

Microscopic Observations of the Wounds. Microscopic examination of the three-day-old wounds of the weanling rats showed that the thickness of the granulation tissue formed was greater in the control than in the

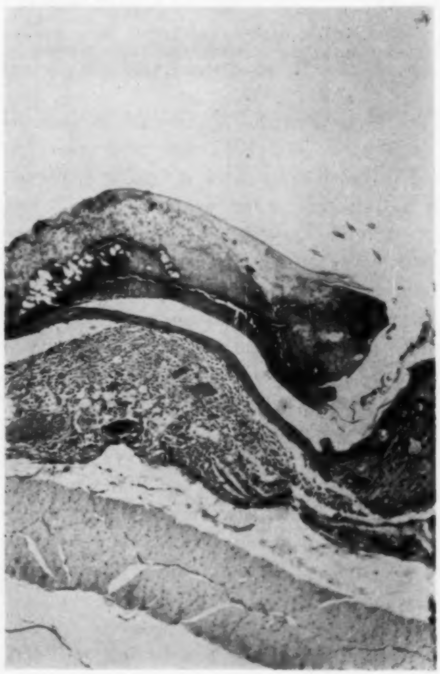
experimental animals (Figs. 4 and 5) and that the number of fibroblasts per unit area was higher in the controls. Comparison of the fibroblasts in the wounds of control and experimental animals revealed those in the controls to be larger, with more abundant cytoplasm and large, ovoid, vesicular nuclei with prominent nucleoli, while those in the experimentals were smaller, with less cytoplasm and smaller, spindle-shaped nuclei with a dense chromatin network (Figs. 6 and 7). No difference in the number of capillaries in the granulation tissue of the two groups of animals could be seen. At this time epithelization had started in both groups. It appeared that the epithelium in the experimental rats had extended a greater distance than in the controls.

Microscopic examination of the control and experimental wounds led to the impression that the inflammatory cells were more numerous in the controls. They were frequently present in large numbers in the underlying muscular layers. This extension of inflammatory cells into the underlying tissues was never observed in the experimental rats. The tissue elements were more widely separated in the control than in the experimental wounds, suggesting that edema might have been more intense in the former than in the latter.

In the nine-day-old wounds of the weanling rats no great differences in the number and morphology of the fibroblasts could be observed. If there was any difference, in the experimentals the fibroblasts were fewer per unit area and more spindle-shaped. The number of collagen fibers per unit area was much lower in the experimentals. In addition, foci of an eosinophilic material containing fine disoriented fibrils were frequently seen in the experimental animals. Large numbers of red blood cells were al-

Fig. 4.—Granulation tissue of three-day-old wound of a weanling control rat (pair-fed control of rat of Fig. 5). Hematoxylin and eosin; × 60.

Fig. 5.—Granulation tissue of three-day-old wound of a weanling rat receiving 0.4% β-aminopropionitrile fumarate. Its thickness is much smaller than that of the control rat. Hematoxylin and eosin; × 60.



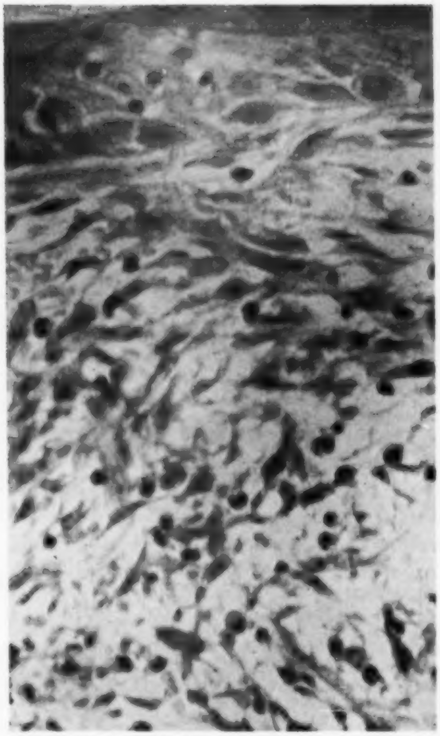


Fig. 6.—Granulation tissue of three-day-old wound of a weanling control rat (pair-fed control of rat of Fig. 7). Hematoxylin and eosin; × 430.

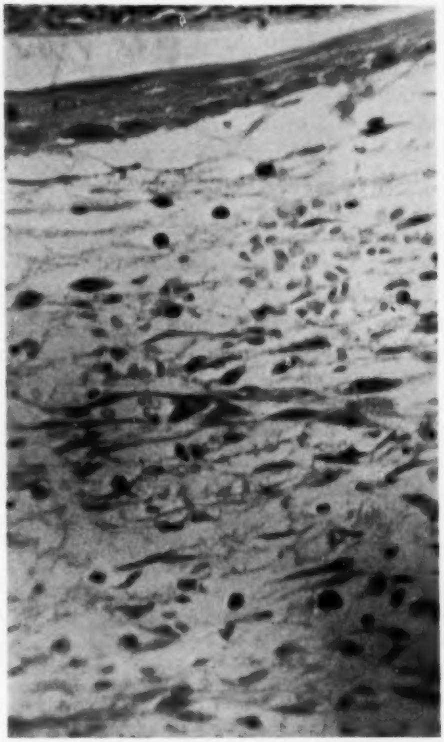


Fig. 7.—Granulation tissue of three-day-old wound of a weanling rat receiving 0.4% β-aminopropionitrile fumarate. As compared to its control, the fibroblasts are fewer per unit area, are smaller, and have less cytoplasm, and their nuclei are smaller, spindle-shaped, and have a dense chromatin network. Hematoxylin and eosin; × 430.

ways present in the wounds of the experimental rats. These cells were well preserved and in most cases were diffusely dispersed in the stroma of the wound (Figs. 8 and 9). In addition, the red blood cells were occasionally found in cyst-like spaces surrounded by spindle-shaped fibroblasts. In the cut edge of the panniculus carnosus the fibroblastic proliferation was more intense in the experimental rats. In this location the fibroblasts of the control rats had a tendency to be more spindle-shaped (Figs. 10 and 11). In both groups the wounds were completely covered with epithelium.

No differences between experimental and control groups were observed in the three-day wounds of the adult rats.

In the 12-day-old wounds of the adult experimental rats, differences from the controls were confined to the granulation tissue in the central and superficial part of the wound. This consisted of the presence of cyst-like spaces containing large numbers of red blood cells and surrounded by immature fibroblasts. In these areas the number of collagen fibers was smaller than in the controls and foci of eosinophilic material similar to those seen in the weanling rats were observed (Figs. 12 and 13).

Comment

Since the gross and microscopic changes were more pronounced in the wounds of the weanling rats than in the wounds of the adults, the changes observed in the weanling rats will be discussed first.

Fig. 8.—Granulation tissue of a nine-day-old wound of a weanling control rat (pair-fed control of rat of Fig. 9). Hematoxylin and eosin; × 430.

Fig. 9.—Granulation tissue of a nine-day-old wound of a weanling rat receiving 0.4% β-aminopropionitrile fumarate. As compared to the control, the number of collagen fibers per unit area is markedly decreased, the fibroblasts are smaller, are spindle-shaped, and have dense nuclei. Numerous extravasated red blood cells are present. Hematoxylin and eosin; × 430.

The observations in the wounds of the weanling rats suggests that the administration of β-aminopropionitrile suppresses wound contraction and granulation-tissue formation but does not seem to affect epithelization significantly.

In the experimental weanling rats the process of contraction was markedly reduced (Figs. 2 and 3). Although the mechanism of

Fig. 10. — Fibroblastic response at the cut edge of the panniculus carnosus of a weanling control rat (pair-fed control of rat of Fig. 11). Hematoxylin and eosin; reduced 15% from mag. × 100.

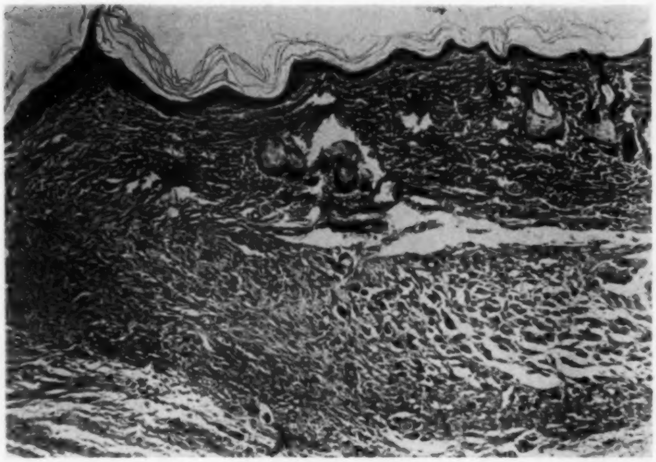


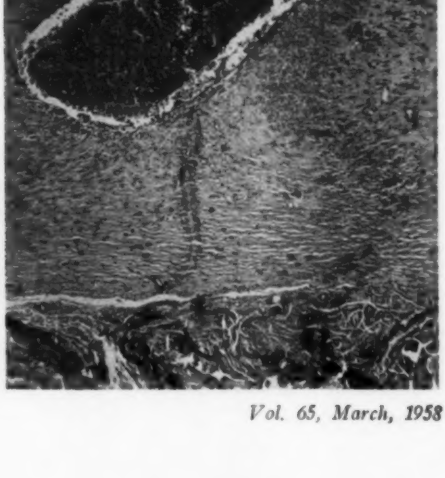
Fig. 11.—Fibroblastic response at the cut edge of the panniculus carnosus of a weanling rat receiving 0.4% β-aminopropionitrile fumarate. The response is more intense than that of its control. Hematoxylin and eosin; reduced 15% from mag. × 100.

contraction is not understood, the consensus is that the force drawing the edges of the wound closer to one another arises in the granulation tissue.[7-11] There is circumstantial evidence suggesting that the collagen fiber may be the source of the contractile force exhibited by the granulation tissue.[10,12]

With respect to the causal relationship between collagen-fiber formation and wound contraction, our observations are of some interest. In the weanling experimental rats the decrease in collagen-fiber formation was accompanied by suppression of contraction,

Fig. 12.—Granulation tissue of a 12-day-old wound of an adult control rat (pair-fed control of rat of Fig. 13). Hematoxylin and eosin; × 60.

Fig. 13.—Granulation tissue of a 12-day-old wound of an adult rat receiving 0.4% β-aminopropionitrile fumarate. A large cyst-like space filled with red blood cells can be seen. Hematoxylin and eosin; × 60.



whereas in the adult experimental rats, in which the greatest part of the wound had healed normally, wound contraction was not affected.

The degree of inhibition of wound contraction in the experimental weanling rats as compared to the wound contraction of their pair-fed controls was not constant throughout the experimental period. At the end of the first three days after wounding the percentage wound contraction in the control and experimental rats was 49% and 26%, respectively, this is approximately two times greater in the controls. During the three- to six-day period after wounding the percentage wound contraction in the control and experimental rats was 54% and 10%, respectively; this is approximately five times greater in the controls. Thus, the inhibitory effect of β-aminopropionitrile upon wound contraction, although evident throughout the experimental period, was more pronounced between the third and sixth days. The percentage wound contraction during the six- to nine day period after wounding was not significantly different in the two groups of animals. This apparent lack of difference in wound contraction between the control and experimental animals during the six- to nine-day period may be attributed to the small size of the control wound on the sixth day after wounding. In this regard, Carrel and Hartmann [7] have shown that contraction is negligible in small wounds. It may be noteworthy to mention that the absence of any significant difference in decrease during the third period occurred in spite of the fact that on the sixth day the wounds of the experimental animals were approximately three times larger than those of the controls. From these observations it is evident that β-aminopropionitrile has a prominent effect during the sixth to the ninth days as well as during the third to sixth. Since the inhibitory effect of β-aminopropionitrile on wound contraction appeared to be greater during the later stages, it may be that wound contraction is due to the action of several factors rather than one. If this is correct, it would appear that those acting on the early stages of wound healing are less affected by β-aminopropionitrile than those acting on the later stages. It has been suggested that wound contraction in the early stages of wound healing may be due to contraction of the fibrin covering the surface of the wound [9] or to cellular migration and remodelling of the tissues (morphyllaxis).[13] It would be interesting to know whether these latter processes are affected by β-aminopropionitrile, but evidence on these points is not available. In regard to the fact that β-aminopropionitrile has its greatest effect in the later stages of healing, it is interesting to note that these animals developed considerably less collagen per unit area and, possibly, therefore, less contraction.

The decreased thickness of the granulation tissue, the small number of the fibroblasts per unit area, the differences in their appearance, and, finally, the decreased number of collagen fibers per unit area suggest that in the experimental weanling animals the formation of granulation tissue is suppressed. The mechanism by which β-aminopropionitrile brings about this effect is not at the present time well understood.

The fibroblastic response of the weanling experimental rats is of interest. There is no difference in the morphology of the fibroblasts of the dermis in animals fed β-aminopropionitrile as compared to the controls. However, once a wound is made the morphology of the fibroblasts that proliferate into the wound of the experimental animals is different from that of the fibroblasts in the wounds of the controls. Furthermore, the response in the experimental animal differs in different parts of the wound. First, in the granulation tissue, the proliferation and differentiation of the fibroblast in response to the stimulus of "wounding" is suppressed as compared to that of the controls (Figs. 6 and 7). Second, in the cut edge of the panniculus carnosus, an area where mechanical stress can be assumed to be present, the fibroblast responds by a more intense proliferation and has a tendency to be more

immature than in the controls (Figs. 10 and 11). We do not know why the morphology of the fibroblastic response in the experimental rats differs in different parts of the wound. However, the tentative hypothesis may be made to the effect that the variation depends upon the nature of the stimulus eliciting the response.

In contrast to our findings, Enzinger and Warner [14] reported increased fibroblastic activity in the wounds of rats receiving aminoacetonitrile for 15 days. The experimental conditions are not similar to ours because aminoacetonitrile was used and because the administration of the nitrile was not initiated until 24 hours after the production of the wounds. Furthermore, the wounds were incisions located in the abdominal wall and may have been subject to mechanical stress resulting from the distention of the abdomen, an occurrence often seen in rats receiving substances with lathyrous activity. Similarly, Mielke et al.[15] have reported that the fibroblasts of the croton oil granuloma pouches of rats receiving β-aminopropionitrile were always more immature than the fibroblasts of the controls. Here too, the conditions are not comparable to those of our experiment because croton oil was used as an irritant and because mechanical factors may enter into the picture, since the walls of the granuloma pouch may be under pressure from the injected air.

In our experimental weanling rats the number of collagen fibers per unit area was found to be smaller than that of their pair-fed controls. This observation is in agreement with the histological findings of Enzinger and Warner [14] and Mielke et al.[15] Coupled with the chemical findings of the latter investigators this suggests a disturbance in collagen formation. Hence, it is apparent that a disturbance of collagen formation is the end-result of the action of β-aminopropionitrile on wounds in rats, even though the morphology of the fibroblasts varies with the various experimental conditions reported.

Our observations suggest that the inflammatory response of the weanling experimental rats may also be suppressed. Selye [16] observed that the volume of exudate accumulating in the granuloma pouches of the experimental rats was less than that of the controls, and Mielke et al.[15] reported that the pouches of the experimental rats were collapsed. These observations suggest that in lathyrism the inflammatory response may be suppressed. The effect, if any, of β-aminopropionitrile on inflammation is at present not well established and needs further investigation, especially since inflammation and repair are two very closely related phenomena.

Geiger et al.[1] were the first to describe hemorrhages (other than those associated with aortic aneurysms) in rats receiving seeds of L. odoratus. Since then, the presence of such hemorrhages has been confirmed [14,17,18] but not adequately stressed. In the wounds of our experimental rats the presence of extravasated red blood cells was a most striking feature in both the weanling and the adult animals. This phenomenon may be the result of a defect of the wall of the small vessels, resulting in increased fragility. Such vascular changes may affect the nutrition of the granulation tissue and may play a role, although not necessarily a primary one, in the disturbance in granulation-tissue formation.

Our experimental methods did not permit quantitation of epithelization because the healing was under a crust. Upon histologic examination, however, the wounds of the weanling experimental and control rats were found to be covered by epithelium on the same day. It may, therefore, be assumed that, in contrast to contraction, epithelization does not seem to be affected by β-aminopropionitrile. This assumption is further strengthened if we consider that owing to the decreased contraction the epithelium in the experimental rats had a larger area to cover than in the controls.

Gross examination of the wounds of the adult rats did not reveal any differences

between the experimental and control animals. The rate of wound contraction was the same in both groups. Similarly, upon microscopic examination the wounds of the adult experimental rats did not exhibit any changes, with the exception, as previously mentioned, of the central, superficial part of the wound; this is the part of the granulation tissue formed latest.

Since granulation tissue is a newly formed connective tissue, it was expected that β-aminopropionitrile would have an equal effect in both weanling and adult animals. Our findings did not substantiate this expectation. It may be that the difference in response is the result of the difference in age of the animals. However, examination of the data on food consumption and body weight of our experimental rats indicate that the weanling rats ingested 52 mg. of β-aminopropionitrile per 100 gm. of body weight per day, while the adult rats ingested 28 mg.† It may, therefore, be suggested that had the dosage been the same the changes in the wounds of the adult animals would have been similar to those of the weanling rats. This suggestion is substantiated by the findings of Enzinger and Warner.[14]

It has been well established that the weight gain of rats receiving β-aminopropionitrile or seeds of L. odoratus is less than that of ad libitum-fed controls. Dasler[19] has reported that the same holds true when the controls are pair-fed. In our experiments, however, there was no significant difference in weight gain between the experimental rats and their pair-fed controls (Fig. 1). This discrepancy may be due to the fact that the control and experimental diets used by Dasler were qualitatively different: Where seeds of L. odoratus were used in the experimental diet, seeds of edible peas were used in the control. Their nutritive values could, therefore, have been different. It should also be considered that the effects of the administration of whole pea may not be exactly duplicated by β-aminopropionitrile. Our findings seem to indicate that the decreased weight gain of rats fed β-aminopropionitrile is the result of decreased food intake. Although the control and experimental rats had the same weight, it appeared upon gross observation at autopsy that the depot fat and muscle mass was greater in the controls than in the experimentals. Data relative to the carcass composition of animals fed β-aminopropionitrile are not at present available. Such studies, and especially nitrogen-balance studies, may prove to be helpful in a better understanding of the pathogenesis of the changes seen in lathyrism, including wound healing.

† The quantity 1 gm. of β-aminopropionitrile fumarate is equivalent to 0.55 gm. of β-aminopropionitrile.

Summary

The wounds of weanling rats receiving 0.4% β-aminopropionitrile fumarate in their diet exhibited the following changes as compared to their pair-fed controls:

(*a*) Wound contraction was markedly decreased, this effect being more pronounced in the later stages of wound healing.

(*b*) The granulation tissue was thinner and showed numerous extravasated red blood cells and foci of an eosinophilic material containing fine, disoriented fibrils. The number of fibroblasts per unit area was decreased, and throughout the experimental period these cells were more spindle-shaped than in the controls. The number of collagen fibers per unit area was also decreased.

(*c*) In the cut edge of the panniculus carnosus the fibroblastic proliferation was more intense in the experimental rats than in their controls.

(*d*) Epithelization of the experimental wounds was not affected.

In the adult rats receiving β-aminopropionitrile, contraction and epithelization were not affected and changes in the granulation tissue were relatively slight.

There was no difference in weight gain between rats receiving β-aminopropionitrile and their pair-fed controls.

Dr. Roger Terry, Department of Pathology, took the photomicrographs and Dr. Arthur Dutton, Statistics Section of the Atomic Energy Project, did the statistical analysis.

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Idiopathic Necrosis of Bone in Small Laboratory Animals

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During a study of the spontaneous skeletal changes that take place with age in small laboratory animals, we have encountered areas of apparently aseptic necrosis of bone with some frequency. So far as we are aware, the lesions have not been described previously. The lesions reported here represent a frequent, sometimes crippling, disorder, and they may be of potential usefulness in the experimental study of such conditions as osteochondritis dissecans, aseptic necrosis of epiphyses, and primary types of bone infarct.

Methods and Materials

The studies previously reported in detail[1,2] included sections of the knees of 670 mice 12 to 22 months old, the hips of 147 of these mice 18 to 22 months old, and the knees and tibiotarsal joints of 144 rats 21 to 30 months old. The skeleton of 122 mice, macerated with papain, have been examined grossly. Sections of several bones of 11 Syrian hamsters 14 to 24 months old and of 8 guinea pigs 29 to 51 months old were examined microscopically.

Results

Mice.—Areas of necrosis were recognized in the sections of bone from 31 of 670 mice—an incidence of 4.6%. There was considerable variation among the strains, as noted in the Table. Among females of the BL/HeN strain, 5 of 23 examined histologically and 5 of 12 papin-prepared skeletons showed the lesions, while certain other strains were not affected at all.

The lesions were usually quite sharply circumscribed and of limited extent. In 14 of the 31 affected animals, the necrosis was confined to the epiphysis (Fig. 1); in another 10, to the metaphyseal region; while in 7 animals combinations of various portions of the femur and tibia were involved. The metaphyseal lesions characteristically were triangular or wedge-shaped, the base lying along the epiphyseal plate. In the earliest stages of the lesion the necrosis had a clearly coagulative character. Pycnosis, fragmentation, and lysis of marrow cells and osteocytes were observed (Fig. 1). The adipose-cell outlines were disrupted. There were occasional minute areas of hemorrhage. At no time was there a purulent reaction. Small numbers of vacuolated plump mononuclear cells, presumed to be lipophages, were present in the necrotic marrow. Calcific granules were deposited in the necrotic fat in a few animals. Brown-Brenn,

Incidence of Bone Necrosis in Inbred Mice Twelve to Twenty-Two Months Old

Strain	Histologic Section, No. Animals*		Papain Maceration, No. Animals†	
	Males	Females	Males	Females
A/HeN	2/16	2/24	0/2	0/0
A/LN	0/20	0/27	0/0	0/0
BALB/cAnN	0/20	0/35	0/2	0/0
BL/HeN	0/18	5/23	0/5	5/12
BLCP/N	0/11	0/18	0/0	0/5
BRSUNT/N	1/21	0/25	0/7	0/6
C3H/HeN	1/15	2/14	0/1	0/0
C3HfB/HeN	0/14	4/24	0/1	0/0
C57BL/6JN	0/22	1/21	0/0	0/1
C57BL/10H-2d	0/11	0/15	0/1	0/3
C57BL/10ScBs	0/10	1/19	0/0	0/0
C57BR/cdJN	0/15	0/20	0/0	0/1
C57L/HeN	0/17	1/24	0/24	0/1
C58/LwN	1/14	1/10	0/0	0/0
DBA/2JN	0/16	0/29	0/19	0/0
STR/N	1/10	0/19	0/5	0/2
STR/1N	1/15	3/25	0/14	0/10
SWR/N	1/14	3/19	0/0	0/0
Total	8/279	23/391	0/81	5/41
Per cent	2.9	5.9	0	12.2

* Number with necrosis/total number animals.
† Number with fractured neck of femur/total number animals.

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From the National Institute of Arthritis and Metabolic Diseases (Dr. Sokoloff) and The National Cancer Institute (Dr. Habermann) of the National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

Fig. 1.—Apparently aseptic necrosis of epiphysis of head of tibia of 13-month-old mouse, Strain A/HeN. Marrow cells have disappeared from the epiphysis but are numerous in the metaphysis of the tibia as well as in the distal femur. Osteocytes are not present in the trabeculae of bone in the epiphysis, but chondrocytes in the epiphyseal plate and articular cartilage are preserved. Hematoxylin and eosin; × 63.

Giemsa, periodic acid-Schiff and Ziehl-Neelsen stains failed to reveal the presence of micro-organisms in the lesions. Although the walls of minute vessels within the lesions were themselves necrotic, thrombus was identified in a nutrient vein in only one instance and in an isolated synovial vessel in the pulvinar acetabuli in one other.

Penetration of endothelial cells and fibroblasts from the viable margins was found in one-third of the animals, and in another third the lesions had become entirely fibrotic, all necrotic-cell detritus having disappeared (Fig. 2). In one instance, the fibrous tissue had undergone calcification and partial bony transformation (Fig. 3). The margin of the fibrotic lesions was frequently delimited by a zone of trabecular new bone formation (Fig. 2). Seams of apposition of new bone were commonly present on the endosteal aspect of the cortex adjacent to the healed lesions. In several instances, healing of small lesions was characterized by a loss of hematopoietic tissue from the marrow and disappearance of osteocytes, but the adipose tissue of the marrow appeared normal (Fig. 3*B*).

When the epiphysis and metaphysis were affected, the chondrocytes of the epiphyseal plate also were necrotic. The articular cartilage, even when the epiphysis was completely necrotic, was spared; the chondrocytes remained viable. Osteoarthritic erosion occurred with the frequency expected in the several strains independently of the necrosis of bone.

In some cases, the necrosis led to complications of several types:

(1) The commonest complication of the necrosis, when the proximal end of the femur was affected, was fracture of the femoral neck (Fig. 4). The head of the femur was frequently displaced within the joint. Severe traumatic arthritis, with

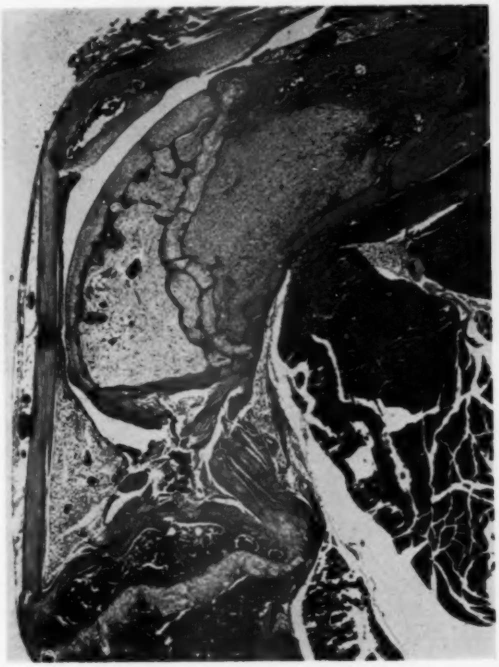

Fig. 2.—Advanced healing of necrosis of femur of SWR/N mouse killed at 19 months of age. The marrow of the distal epiphysis and shaft has been replaced by fibrous tissue of loose texture. The chondrocytes of the epiphyseal plate as well as the osteocytes of the adjacent bone have disappeared. At the proximal margin of the lesion in the shaft of the femur, a rim of new bone has been formed. The articular cartilage is intact. In other bones of this animal, more unhealed areas of necrosis were present. Hematoxylin and eosin; × 16.

fibrosis and fibrocartilaginous and bony metaplasia of the articular and periarticular soft tissues, resulted. The head and neck of the femur were necrotic in a number of instances when no fracture was present, indicating that the necrosis was the primary event rather than the consequence of the fracture.

(2) In one animal, the necrotic epiphysis of the head of one tibia had collapsed (Fig. 5*A*); the articular cartilage was fractured, displaced into the epiphysis, and necrotic (Fig. 5*B*). Marginal callus formation resulted in a bulbous appearance of the knee.

(3) Mention has already been made of the ossific transformation of the metaphyseal lesion in the femur of one animal. The occurrence of bone necrosis and fatty transformation of the marrow of the epiphysis of the head of the tibia of the same knee supports the diagnosis of healed necrosis of bone rather than primary medullary bone tumor.

Aside from genetic factors, a strong sex influence was found—5.9% of all females being affected, compared to 2.9% of males. The lesions were found scattered randomly in all age groups from 12 to 21 months. In 2 of 380 mice, the epiphysis of the medial epicondyle of the humerus was necrotic during the first two months of life. Complete histological examination of the skeleton was made in four affected mice; in two, the head of the humerus was found to be involved in addition to the femur or tibia, while in two others, the lesions were confined to one epiphysis of the hind extremities.

Leukemia was present in only 4 of the 31 mice with bone necrosis. Sections of the viscera were made in four instances. In one of these, coagulation necrosis of viscera infiltrated with leukemic cells was observed. There were no disseminated vascular lesions. Cultures of the lesions were not made.

In addition to these lesions observed in the large bones, a similar process of necrosis was found in a large proportion of all older mice in the small bones of the knee—the

Fig. 3.—Localized osteosclerosis arising from healing of necrotic lesion in distal metaphysis of femur (C3HfB/HeN female, 19 months old). *A*, roentgenogram; a sharply delimited wedge-shaped area of radiopacity is present immediately proximal to the epiphysis of the femur on the left; the contralateral metaphysis is normal. *B*, the corresponding lesion, after decalcification, extends to the epiphyseal plate. It is composed of compact fibrous tissue and membranous bone. The bone of the epiphysis of the head of the tibia has no viable osteocytes; hematopoietic cells have disappeared from the marrow, but the adipose tissue within it is viable. Hematoxylin and eosin; × 18.

lunulae of the menisci and the suprapatellar ossicle that is found in certain strains.

Rats.—Large areas of necrosis have not been found in the strains of rats examined. Rare instances of necrosis in the head of the femur have been observed. Nevertheless, the bony portion of the menisci of the knee of a large proportion of rats in this old age group were necrotic as in the mice.

An additional pattern of necrosis has been observed in association with dorsal thoracolumbar kyphosis of older rats (Fig. 6*A*). Such kyphosis has been recognized as a sporadic lesion of older rats by others, although the pathological changes have apparently not been described. The histologic appearance of one such lesion is illustrated in Figure 6*B*; the proximal epiphysis of

Fig. 4.—Fracture of neck of femur associated with aseptic necrosis (BL/HeN female, 16 months old). Numerous excrescences of bone have formed about the capsular attachments to the intertrochanteric portions of the femora.

two adjacent vertebral bodies in the kyphus has undergone coagulation necrosis like that described above. The remainder of the vertebral bodies in this region as well as the hindlegs were not affected by the process.

Hamsters.—In one hamster, one talus was completely necrotic except for its articular cartilages.

Guinea Pigs.—Necrosis has not been observed in the small number of animals studied.

Comment

The etiology of the lesions described is obscure. They appear to be aseptic and have many of the characteristics of infarcts. Despite this fact, occlusion or inflammation of the nutrient or periarticular arteries has not been apparent. The lesions thus appear to resemble several patterns of necrosis of bone that occur in man—osteochondritis dissecans, primary bone infarcts, and the various juvenile osteochondroses. Bragdon reviewed the literature on the subject of experimental production of bone infarcts in laboratory animals and found that the general experience in human material and animal experiments was that ordinary occlusion of the nutrient arteries of bone did not account for the development of bone infarction. The reason offered for this was that the vessels supplying the bone (nutrient, periosteal, and epiphyseal arteries) anastomose freely, particularly in the adult. Bragdon did succeed in producing small areas

Fig. 5.—Unilateral collapse of tibial epiphysis, C3HfB/HeN female, 19 months old. *A*, roentgenogram; the condylar end of the femur lies within a large concave deformity of the head of the tibia. *B*, articular surface of collapsed tibia. Necrotic spicules of bone are present in granulation tissue that replaces normal marrow. Most of the chondrocytes of the articular cartilage are dead, and the depressed cartilage lies at the surface of the granulation tissue. Hematoxylin and eosin; × 175.

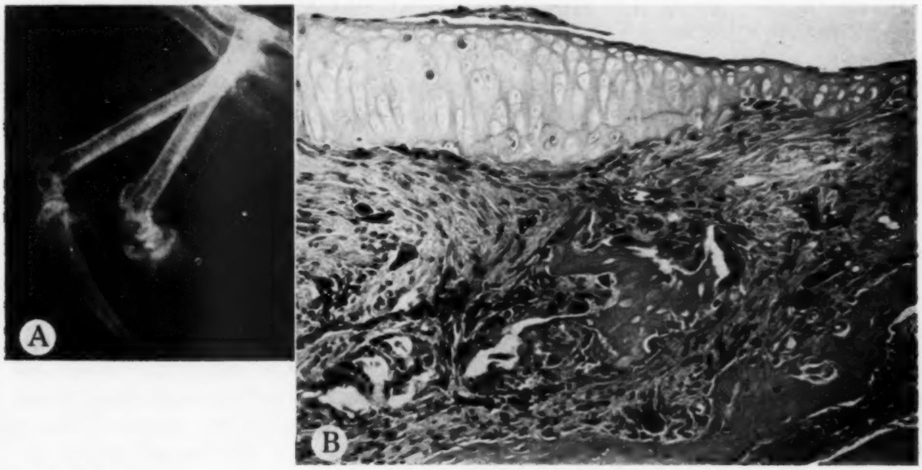

Fig. 6.—*A*, thoracolumbar kyphosis of 20-month-old OM/N rat. The hump was first noted several weeks before this photograph was taken. *B*, sagittal section of vertebral body in preceding kyphus. Marrow cells and osteocytes in the proximal epiphysis have disappeared. Hematoxylin and eosin; × 30.

of infarction in the bones of young rabbits by cutting the nutrient artery to the femur.[3] Old lesions resembled some of those observed in the present series—those in which there were circumscribed areas of loss of hematopoietic tissue from the bone marrow without dissolution or replacement of the adipose tissue. Zemansky and Lippman[4] succeeded in producing ischemic necrosis of the head of the femur of rabbits 2 weeks old by interrupting the vessels in the ligamentum teres. This was not successful in animals 7 weeks old, at which time the vessels disappear spontaneously. Vessels were not seen at 60 days of age in the ligamentum teres of five inbred strains of mice that we have examined, although special injection methods were not employed. The avascularity of this ligament makes it unlikely that embarrassment of blood supply of this source is a factor in the development of the necrosis observed in the head and neck of the femur of mice.

Mau produced aseptic necrosis in the caudal vertebrae of rats 6 to 7 weeks old by twisting their tails beneath the abdomen and fixing them in this position.[5] After 80 to 120 days the marrow and bone of the

proximal epiphyses in the region of the concavity were acellular. Vascular changes were not seen, but it was surmised that a circulatory disturbance had in fact been present—that the small nutrient vessels passing through the thick bone were compressed by the deformity. The appearance of the lesion in the kyphotic rat is strikingly similar to that produced by Mau, and it may be that the kyphus exerted a stress comparable to that of his experiment. The genesis of kyphosis in old rats is not established. It has been referred to in the German literature as *Haltungskyphose* (cage kyphosis).[6] This term implies that it has a postural basis, arising from the enforced crouched position in low cages.

An infectious etiology cannot be ruled out because appropriate cultures were not made. Nevertheless, organisms could not be identified after careful microscopic examination of the sections. The bacteria that are most often recognized to affect the skeletal system of mice—Corynebacterium kutscheri and Streptobacillus moniliformis—are stated to be recognized without difficulty in sections.[7-9] The character of the necrosis without inflammatory-cell infiltration suggests an ischemic rather than infectious process.

In mice, genetic and sex influences appear to be involved. The incidence varies from as high as 22% in females of one strain (BL/HeN) to 0 in others. Twice as many females as males are affected. Histological studies of the viscera of the BL/HeN mice were not made, and we have no knowledge of the occurrence of vascular disease in them. Old breeder female BL/HeN mice, maintained separately from the present ones for five years in the laboratory of Dr. Margaret Deringer, are prone to develop necrotizing arteritis in the abdominal and pelvic organs (personal communication from Drs. Thelma Dunn and Margaret Deringer). Unlike our animals that received Purina Fox Chow, the latter are fed Morris' diet (manufactured by Herbert Bryant Mills, Gaithersburg, Md.). The hips of 10 of Dr. Deringer's mice were studied histologically. Although iliac arteritis was present in several, there were no instances of necrosis of bone. It has already been noted that vascular lesions were not apparent in the sections of necrotic bone. So far as we are aware, the animals have not been subjected to toxic substances or traumatic handling. Their diet has been a standard one and has sufficed to maintain the animals through a normal life span and reproductive period. Dr. M. Silberberg has observed the lesions in older mice infrequently and has not found its incidence affected by various diets (personal communication).

Because of the frequent complications by fracture of the neck of the femur, the lesion must be regarded as a serious disorder of animals in the strains where it occurs in high incidence. In other locations, it apparently may heal without serious consequence. Because of the pathological similarity to the several types of aseptic necrosis of bone that occur in man, these animals may provide a tool for future study of these diseases. Similar lesions have also been found occasionally in young swine.[10]

Summary and Conclusions

Localized areas of spontaneous, apparently aseptic necrosis of bone have been observed in 31 of 670 mice during the second year of life. The etiology is obscure, but the lesions appear to be influenced by genetic factors and sex. Isolated instances occurred in rats and a hamster. The lesions at times healed completely or were complicated by fracture of the femoral neck, collapse of epiphyses, kyphosis, or localized osteosclerosis simulating a bone tumor. The ossicles of the knees of a large proportion of all older rats and mice studied had a comparable pattern of necrosis.

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The Significance of the Increase of Nonspecific Lipid Components in Primary Lipoid-Storage Diseases

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It is well recognized that the primary lipoid-storage diseases present an opportunity to derive information concerning normal human intracellular lipid metabolism.* This is especially true in view of the possibility offered with respect to the isolation and study of previously unknown lipids, inasmuch as these may be concentrated in large amounts in the involved organs while escaping detection in the normal because of the relatively small amounts in which they occur in tissues. Thus, our knowledge of the existence of gangliosides stems from Klenk's original isolation of this water-soluble glycolipid in Tay-Sach's disease,[1,2] while the existence of polycerebrosides was first demonstrated in Gaucher's disease,[3] although it has since been recognized that both constitute normal components of almost every organ in the body.[2,3]

It is perhaps for these reasons that all textbook accounts of the chemical pathology of primary lipoid storage diseases stress the specificity that labels the clinical entity in chemical terms, so that Gaucher's disease is known by its accumulation of cerebrosides and polycerebrosides; Niemann-Pick's disease, by the enrichment in sphingomyelin, and Tay-Sachs' disease, by the accumulation of gangliosides.[4] The biased emphasis on this apparent chemical specificity that the primary storage diseases enjoy has obscured other pertinent analytical data, leading to an oversimplified formulation of each of these diseases as being due to a genetically determined intracellular deficit in the enzymes responsible for orderly metabolism of naturally occurring lipids, resulting in a specific enrichment of these in each of the particular diseases concerned.[4] Yet the most cursory examination of published analytical data on the lipid constituents in cases of primary lipoid disorders indicates that, quite aside from the increase (as per cent of the total lipid) of the specific lipid fraction that characterizes a disease, there is also a marked increase in the total lipid content of the organs involved, resulting in an absolute increase of almost all lipid fractions. In other words, the increase in the specifically stored lipid does not account for the increase in the total lipid content of the involved tissue.

This fact was repeatedly pointed out by earlier workers [4] and emphasized by me, not only for the primary lipoid-storage diseases but also for other types of storage diseases, like gargoylism.[5] The fallacy engendered by the attraction of the concept of a specific enzymatic block or deficiency appears to dominate most of the thinking in this field, with a resulting lack of concern for the necessity of explaining the increase in "nonspecific" lipid fractions in so-called specific storage diseases.

The present report attempts to document further the nature of the increase in all lipid

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* Throughout this paper the terms "lipid" and "lipoid" are not used as synonymous. The word "lipid" designates a chemical class including all derivatives of fatty acids, while "lipoid" is used in the broader sense as a descriptive term for substances that appear to be "fat-like" in their histological and histochemical characteristics though not necessarily identical with "lipid."

fractions, quite aside from the specific increase of the particular lipid characterizing Gaucher's and Niemann-Pick's disease, in cases studied personally by me and to explain this general phenomenon in terms of an altered, secondary, lipid-binding property that the tissue acquires by virtue of the initial binding of a specific lipid.

There are a number of reasons for restricting the scope of the present study to the lipids and lipoproteins of spleen. Firstly, the human spleen possesses a rather constant composition with respect to lipid constituents. Secondly, the spleen is uniformly and maximally involved in the primary lipoid storage diseases, so that the increase in lipid is usually directly proportional to the number of storage cells. Thirdly, it does not undergo significant secondary changes in lipid metabolism and composition as a nonspecific response to nutritional, toxic, or congestive processes. This factor, on the other hand, renders the liver quite unsuitable for precise studies on lipid accumulation due to a specific lipid disorder because of the constant flux in liver lipid constituents with diet, inanition, fatty degeneration, and fatty metamorphosis that may accompany primary disease processes in a nonspecific manner. Finally, fresh samples of surgically removed spleen were available, which is naturally preferable when it becomes imperative to exclude errors introduced by autolytic changes in autopsy material, which might otherwise cast doubt on the unequivocal nature of results.

It is also obvious that the large amount of lipid normally present in brain precludes a similar study in Tay-Sachs' disease, where the accumulation of lipids in abnormal amounts is restricted to nerve-cell bodies. Since the latter would constitute less than 1% of the total mass of the brain, the documentation of differences in each of the lipid fractions, in addition to the gangliosides, would be a priori an impracticable task. For these reasons Tay-Sachs' disease (and its variants) will not be considered here even though it constitutes a primary lipoid storage disease.

Material

Except for one instance where formalin-fixed spleen was used (Case 3, Table 1), all the material was available in the fresh-frozen state. In most instances, the material was obtained surgically and immediately frozen after sufficient blocks had been removed for routine and special histological examinations. The diagnosis was unequivocally established in each case by the histologic criteria. Data for "normal" spleen were obtained by analysis of eight surgically removed spleens (five hemolytic anemia, one Banti's syndrome, one traumatic rupture, and one hypersplenism syndrome) and three autopsy specimens removed from patients dying from causes unrelated to the lipoid storage diseases (ages 6 to 16 years). The lipoprotein of Gaucher's disease was isolated from the surgically removed spleens of three patients with Gaucher's disease of the chronic type (Cases 4, 6, 8) and designated Ppn. I, II, and III, respectively.

Suitable material from infants without lipid storage disease was not available, hence the data presented for infantile Gaucher's disease and Niemann-Pick's disease perhaps cannot be compared with the "normal" figures given in Table 1, although there appears to be no justification, on histological grounds, to assume that the figures would fall outside the ranges given for an older age group (6 to 16 years).

Methods

Extraction of lipids was performed on 50 to 100 gm. of tissue by homogenizing the tissue with 20 vol. per gram of tissue of chloroform-methanol (2:1 vol. parts) in a Waring Blendor. The homogenates were boiled for three minutes on a water-bath; the extract, filtered off, and the residue, reextracted twice with boiling chloroform-methanol. The combined filtrates were made up to a known volume with chloroform-methanol (total lipid extract). Aliquots of this total lipid extract were freed of nonlipid impurities by the method of Folch et al.,[6] aliquots of this "washed" lipid extract being used for the determination of cerebrosides, total phospholipids, sphingomyelin, and cholesterol. Aliquots of the original total lipid extract were taken to dryness in preweighed evaporating dishes, first under a stream of nitrogen and then in high-vacuum in a desiccator, and the weight of the dried residue thus obtained was used in the calculation of the total lipid content of organ. The reason for using aliquots of this extract rather than the "washed" extracts for determination of total lipid was two-

fold. Firstly, the procedure of Folch et al. results in an almost total loss of water-soluble glycolipids of the ganglioside class [2,6] and the polycerebroside class.[6] Secondly, the amount of contaminants removed by the washing procedure was found, after allowance was made for the loss of water-soluble glycolipids, to amount to less than 1% of the total lipid.

Total lipid phosphorus was determined on the washed lipid extract by the method of Sperry,[7] and total phospholipid, calculated by multiplication of the lipid phosphorus content by a factor of 25. Sphingomyelin was determined in the washed lipid extract by the procedure of Schmidt and Thannhauser.[8] The value for "lecithins plus cephalins" was calculated as the difference between total phospholipid and sphingomyelin. Total cholesterol was determined by the modification of the Schoenheimer-Sperry method.[9] Cerebrosides were determined by both the orcinol method of Brückner [10] directly on the washed lipid extract, as well as on the product of hydrolysis (2 N HCl for two hours) of the total lipid residue with the reductimetric method of Folin and Malmros,[11] the results being expressed as galactose equivalents in both instances. The cerebroside content was calculated from the galactose figures by multiplying with the factor 4.6.

The original total lipid extract was used for the determination of neuraminic acid according to Klenk and Langerbeins [12] in earlier experiments and by the method of Böhm et al.[13] in later studies. Since there is no unanimity as to the neuraminic acid content of gangliosides and since the latter also appear to be lipids inhomogeneous as to composition, no attempt was made to calculate ganglioside content of the total lipid extracts, and only the analytical results on neuraminic acid determinations are reported.

The determination of total water-soluble glycolipid was carried out in the following manner. Total lipid extracts of known lipid content were taken down almost to dryness in a water bath at 70 C. The moist white residue was taken up in a known volume of chloroform, in which it dissolved wholly, and partitioned against an equal volume of cold 4% trichloracetic acid containing 1% NaCl. The latter was found necessary to effect a clear aqueous phase without a thick emulsion layer between itself and the chloroform layer. The salt also served to extract the water-soluble glycolipids from the hydrated cerebrosides packed at the interphase of the two solvents.[14] Aliquots of the aqueous upper layer were used for the determination of hexose by a modification of the method of Brückner [10] or, after hydrolysis, with 2 N HCl, by the reductimetric method of Folin and Malmros.[11] The difference between the glucose equivalent values obtained by the Folin-Malmros procedure on samples before hydrolysis and after hydrolysis was considered to represent the water-soluble glycolipid hexose (in glucose equivalents). Results were expressed as glucose equivalents, since both the gangliosides and the polycerebrosides contain a mixture of glucose and galactose in their compositions.[2,6] Other aliquots of the upper aqueous layer of the partition system (*a*) were used directly for the detection of free glucose and galactose chromatographically [6] and (*b*) were hydrolyzed for two hours with N H_2SO_4; after removal of the acid with excess barium carbonate, the hexose moieties of the water-soluble glycolipids were identified chromatographically, and after elution from the chromatograms, estimated analytically by virtue of their respective reducing values.[6]

The lipoprotein of Gaucher's disease (GLP) was isolated, with minor modifications, according to the procedure previously described.[15] Prior to final purification, that fraction which contains the GLP in complex form with phosphatides was also prepared separately by neutralizing the extract and dialyzing against 0.15 M NaCl. Both the purified GLP and the GLP containing large amounts of phosphatide (GLP-phosphatide complex) were kept in the hydrated state at 0 C and not allowed to dry. Aliquots were removed for analysis to establish their respective identities and compositions.[15]

Lecithin sols were prepared from highly purified cattle-brain and egg-yolk lecithins in the following manner. Extraction and purification of lecithins from the two stated sources was carried out in the conventional manner. After exhaustive extraction with acetone, the residue was extracted exhaustively with boiling ether (20 ml. per gram of acetone residue). The ether extracts were reduced in volume in vacuo until the first precipitate made its appearance, and the phosphatides were precipitated out by the addition of 3 vol. of cold acetone. The precipitates were collected by filtration, redissolved in ether, and the cadmium salts formed by addition of 2% cadmium chloride. Crystallization of the cadmium lecithinate was initiated by adding acetone to the ether solutions until slight turbidity appeared. Crystallization was allowed to continue at 0 C for three days. The heavy yellow precipitate of cadmium lecithinate crystals was recovered by filtration. The cadmium salts were suspended in methanol and treated with ammonium carbonate. Ammonia was removed from the methanolic lecithin solutions in vacuo, and the free lecithin was redissolved in large amounts of ether and the cadmium salt purification repeated three times. To remove the last traces of "cephalin," 10% methanolic solutions of the free lecithin were allowed to stand 24 hours at −10 C. The ensuing precipitates were centrifuged off at the same temperature and the supernatants taken

to dryness in a stream of nitrogen. The residue was redissolved in methanol and the low temperature fractionation repeated three times. Ten milligram aliquots of these lecithin preparations failed to show any measurable α-amino nitrogen upon hydrolysis in sealed tubes under nitrogen at 110 C for six hours, nor could ethanolamine or serine be detected chromatographically, indicating that all cephalins had been removed (final yield, 27% of the original crude lecithin).

For the preparation of sols, the lecithin preparations were dissolved in ether, and these solutions were transferred into 10 vol. of methanol and immediately dialyzed exhaustively in the cold against distilled water. Aggregates that flocculated out were centrifuged off, and the lecithin sols thus obtained were further cleared by 30 minutes centrifugation at 20,000 g. The number-average molecular weight of lecithin micelles was 25,000 as estimated by the osmotic pressure of the solutions as measured in a Zimm osmometer, in which NaCl and Na acetate buffer at pH 7.1 (0.1 M) was employed as the equilibrating solvent. The osmotic pressure of concentrations of 0.1%, 0.2%, 0.3%, and 0.4% lecithin sols were plotted to give the (P/C,C) curve, the extrapolation of which to infinite dilution was used to calculate the number-average aggregate size in the usual manner. Diffusion studies in the Perkin-Elmer Tiselius apparatus against distilled water at 20 C indicated an average micelle weight of 20,000 for most preparations (2.0 ml. cell). Usually, the lower the initial concentration of lecithin in the methanol prior to dialysis against water, the more uniform and stable was the micellar sol formed.* The concentration of lecithin in each preparation of lecithin sol was estimated from the phosphorus content of the solution or by taking aliquots to dryness in preweighed evaporating dishes. The preparations thus obtained were stored at 2 C until further use. The iodine number of unsaturated lecithin sols used in this study ranged between 30.0 and 34.0. Fully saturated lecithins were prepared by the conventional method of hydrogenation at room temperature in methanol either with palladium or nickel catalyst.

Sphingomyelin solutions were similarly prepared after highly purified sphingomyelin had been obtained from cattle brains according to Klenk.[1] These solutions were, however, unstable at concentrations above 0.4% and could not be stored for periods longer than a week.

Dialysis equilibrium experiments were used to demonstrate the loss of residual organic anion-binding power after lecithins or sphingomyelin was bound by the Gaucher lipoprotein. The experimental design for these studies was the same as previously employed.[16] Instead of sodium chloride, however, 0.1 M barbital (Veronal) acetate buffer (Michaelis' buffer) was used in most of the experiments, the amount of residual dye in the outside solution being used as the measure of the binding power of the protein-lipid solution within the dialysis bags. Dye-binding by the viscose tubing and diffusion-dilution effects were compensated for by subtracting the values obtained in blanks containing only buffer within the dialysis bags from the values of the test samples. GLP concentration within the bags was kept constant at 0.2% to obviate corrections for the Donnan effect. When p-aminobenzoic acid was used, either to study the release of already bound dye, or to block the binding of dye, this was added to the outside solution in appropriate amount.

The methyl orange used was a product of the National Aniline Division of the Allied Chemical and Dye Company and was recrystallized twice from aqueous ethanol before use.

Results

As is evident from Table 1, the tissue lipids in both Gaucher's and Niemann-Pick's disease were characterized not only by an increase in cerebrosides and sphingomyelin, respectively, but also by a nonspecific increase in phospholipids, water-soluble glycolipids, and cholesterol. The disparity between the increase in cerebrosides in the spleen in Gaucher's disease and the increase in the other lipid components was most striking (Cases 1 to 8). The same increase in nonspecific lipid components in Niemann-Pick's disease, as contrasted with the specific increase in sphingomyelin, was also illustrated by the findings in Cases 9 to 11. It appears that cerebrosides are much less elevated in proportion to other lipid fractions in Niemann-Pick's disease, whereas there is a considerable elevation in sphingomyelin content in Gaucher's disease pari passu with an over-all increase in phosphatides. It should also be pointed out that the two cases of infantile Gaucher's disease (Cases 1 and 2) presented a much more pronounced increase in water-soluble glycolipids than in cerebrosides (water-insoluble glycolipids). This does not appear to be true for the juvenile and adult forms of Gaucher's disease (Cases 3 to 8). What is probably of equal impor-

* The kinetics of micelle formation will be treated in a separate communication to be published elsewhere.

TABLE 1.—*Composition of Lipid Constituents of Spleen*

Case No.	Normal*	Gaucher								Neimann-Pick		
		1	2	3	4	5	6	7	8	9	10	11
Age		3 mo.	5 mo.	2 yr.†	7 yr.	9½ yr.	11 yr.	20 yr.	45 yr.	5 mo.	2 yr.	?
Total lipid, % fresh weight	2.68 (2.01–3.17)	3.10	8.6	9.11	9.6	6.42	11.8	12.4	8.01	7.72	15.45	9.82
Total phospholipid, % total lipid	42.68 (39.64–44.92)	31.6	39.4	38.4	39.2	36.8	39.6	42.2	46.0	52.58	65.8	62.4
Sphingomyelin, % total phospholipid	19.96 (12.42–21.8)	15.4	13.3	26.2	20.1	11.4	19.6	16.8	12.6	55.8	69.4	67.2
Lecithins+cephalins, % total phospholipid	80.8 (80.11–87.2)	85.6	86.7	73.8	79.9	88.6	80.4	83.2	87.4	44.2	30.6	32.8
Cerebrosides, % total lipid	0.94 (0.42–1.11)	2.72	14.42	4.64	7.62	8.64	9.65	11.46	17.8	1.2	1.74	1.66
Water-soluble glycolipids (ganglioside+polycerebroside) as glucose equivalents, % total lipid	1.26 (1.11–1.96)	4.6	14.44	2.62	1.99	4.68	2.04	2.11	1.78	12.22	14.04	11.06
Neuraminic acid, % water-sol. glycolipid hexose	12.2 (10.1–14.9)	14.6	16.84	9.46	14.64	10.22	15.44	10.42	9.86	16.84	17.62	18.92
Cholesterol, % total lipid	4.22 (3.68–5.22)	5.61	6.42	7.21	6.73	5.81	5.94	4.97	5.86	6.63	7.11	7.62

* Values in parentheses represent the variation range encountered in surgically removed spleens in patients without lipoid disease.
† This material was only available in formalin-fixed state and was analyzed 4 years after surgical removal and formalin fixation.

tance is the increase in water-soluble glycolipids in the three cases of Niemann-Pick's disease (Cases 9, 10, and 11), in spite of the minimal levels of cerebrosides detected. The possibility that incomplete or artifactual partitioning may have occurred with resultant determination of cerebrosides as water-soluble glycolipids in the aqueous phase of lipid extract partitions is rendered unlikely by the fact that there is a concomitant increase of neuraminic acid in the aqueous phase, suggesting that the water-soluble

TABLE 2.—*Residual Organic Anion-Binding Capacity of the Gaucher Lipoprotein (GLP) and the Complexes It Forms with Lecithin Sols**

	Experiment			
	1†	2‡	3§	4‖
Section A				
Ppn. I GLP	60.7	42.8	39.8	4.2
GLP-phosphatide complex	10.3	6.2	5.7	1.7
Ppn. II GLP	63.4	51.4	50.2	1.9
GLP-phosphatide complex	10.6	6.0	7.2	1.8
Ppn. III GLP	59.6	48.4	49.6	2.6
GLP-phosphatide complex	6.8	5.4	5.7	2.0
Section B				
Lecithin Sol. A 0.2%	2.59	0.68	0.64	..
Lecithin Sol. B 0.2%	2.17	0.62	0.58	..
Lecithin Sol. C 0.2%	3.64	0.74	0.68	0.21
Lecithin Sol. C 0.1%	2.62	0.65	0.60	0.11
Lecithin Sol. C 0.05%	1.99	0.52	0.44	0.05
Section C				
Ppn. I				
GLP+lecithin C 0.01%	65.9	58.6	56.4	4.6
GLP+lecithin C 0.03%	46.5	39.4	38.0	4.2
GLP+lecithin C 0.05%	36.2	26.6	27.0	3.4
GLP+lecithin C 0.08%	34.9	24.0	22.0	5.5
GLP+lecithin C 0.10%	20.7	9.4	10.2	2.2
GLP+lecithin C 0.20%	5.2	2.0	4.2	4.2
Ppn. II				
GLP+lecithin C 0.05%	65.4	49.5	48.5	7.9
GLP+lecithin C 0.08%	39.6	27.2	26.0	6.4
GLP+lecithin C 0.10%	26.2	10.2	12.4	4.6
GLP+lecithin C 0.20%	11.2	5.6	7.2	4.4
GLP+lecithin B 0.20%	9.6	4.2	4.6	5.2
GLP+lecithin A 0.20%	8.4	4.4	5.0	4.2
Ppn. III				
GLP+lecithin A 0.1%	26.4	16.6	15.5	..
GLP+lecithin A 0.2%	15.6	7.5	5.7	3.4
GLP+lecithin B 0.1%	35.2	24.4	26.2	..
GLP+lecithin B 0.2%	11.2	6.2	6.0	3.6
GLP+lecithin C 0.1%	32.6	19.4	21.4	..
GLP+lecithin C 0.2%	12.2	6.8	6.2	3.8

* GLP inside dialysis bags: 20 mg. in 10 ml. of 0.1 M barbital acetate buffer (pH 6.9). Lecithin sols dialyzed against same buffer and added from 1.0% stock solution. The natural GLP-phosphatide complexes were obtained from the same spleens from which the GLP preparations were isolated. Additions of methyl orange (MeOr), *p*-aminobenzoic acid (PABA), and *p*-aminosalicylic acid (PASA) to 20 ml. of outside solution.

† After addition of 140×10^{-7} M to system. MeOr bound $\times 10^{-7}$ M.

‡ Moles ($\times 10^{-7}$) MeOr released by adding PABA 730×10^{-7} M.

§ Moles ($\times 10^{-7}$) MeOr released by adding PASA 650×10^{-7} M.

‖ Noncompetitive binding. Moles ($\times 10^{-7}$) MeOr bound after 730×10^{-7} PABA added to system.

glycolipids measured are probably represented by gangliosides and polycerebrosides.

Dialysis equilibrium experiments designed to measure the avidity for organic anion binding as a factor for the binding of lipid organic anions such as lecithins and sphingomyelin reveals that the previously demonstrated high organic anion affinity of the GLP [15] is operative in vitro in binding lecithins. As is evident from Table 2, the addition of lecithin sols in increasing amounts to 20 mg. of GLP causes a progressive decrease in the amount of sites available for organic anion binding, so that less and less methyl orange is bound with increasing amounts of lecithin present (Table 2, Section C), although the lecithin sol itself, in this experimental circumstance, possesses no significant binding capacity for methyl orange per se (Table 2, Section B). Similarly, naturally occurring complexes between GLP and phosphatides (the GLP-phosphatides complex) possesses less methyl-orange-binding capacity than the GLP which has been purified by dissociating "contaminating" phosphatides. Where competition for the organic anion-binding site was instituted [17,18] with use of a competing carboxyl anion (*p*-aminobenzoic acid and *p*-aminosalicylic acid) versus the sulfonic acid group of methyl orange, interesting observations were made. As illustrated in Table 2, *p*-aminobenzoic acid (PABA) added after equilibrium has been attained in the binding of methyl orange releases only a portion of the methyl orange. The per cent of methyl orange released decreases with the increase of concentration of lecithin solution within the dialysis bag. On the other hand, if *p*-aminobenzoic acid is added to the system before methyl orange, it becomes evident that the binding sites on the GLP are blocked by the PABA, since only very small amounts of methyl orange are additionally bound in these cases. Experiments in which sphingomyelin solutions were used to form complexes with GLP in vitro produced less clear-cut results, although some degree of interference in the additional binding of methyl orange was still evident. This was probably due to the instability of sphingomyelin solutions already referred to earlier.

Comment

The findings presented here, showing an increase in all lipid constituents in cases of Gaucher's disease and Niemann-Pick's disease, agree with the experience of earlier workers. The points that bear comment are the absolute increase of nonspecific lipids, with a relative increase in the cerebrosides in Gaucher's disease and sphingomyelin in Niemann-Pick's disease. Thus, for example, although the percentage changes in lecithins, cephalins, and cholesterol are not significantly increased with respect to the total lipid, the total amount of each lipid fraction present in the organ is quite elevated. The curious maintenance of the relative proportion between cholesterol and phospholipids in the 11 spleens studied invites the conclusion that the increase in phospholipids predisposes to an increase in cholesterol (and presumably neutral fat). Thus, the phosphatide-cholesterol ratio ranges between 5.32 and 8.53 for the Gaucher spleens and between 7.99 and 9.25 for the Niemann-Pick spleens, compared with an average normal ratio of 10.1. The reason for the lower ratio in the Gaucher cases is probably explained by the larger proportion of cerebrosides and polycerebrosides present, so that the aliphatic side-chains of the glycolipids act as the lipophilic cholesterol-binding sites, which are otherwise provided in the normal and in Niemann-Pick's disease by the fatty acid residues of the phosphatides. The fact that the spleens in the infantile form of Gaucher's disease (Cases 1 and 2) were found to be low in cerebrosides but relatively highly enriched with water-soluble glycolipids of the ganglioside and polycerebroside class can be considered to confirm the previously proposed hypothesis that the water-soluble glycolipids constitute precursors of the water-insoluble cerebrosides.[2,3] The hitherto unrecognized increase

of water-soluble glycolipids in the infantile form of Gaucher's disease, with relatively little increase in cerebrosides, may explain the high "glucocerebroside" values reported in this form, while the adult form is generally accepted to show the more orthodox galactocerebroside accumulation.[4] The high glucose content of water-soluble glycolipids would be expected to account for the high "glucocerebroside" value when analyses are carried out directly on tissue lipids without prior isolation of pure cerebroside. That the water-soluble glycolipids here measured do not belong exclusively to the ganglioside group is attested to by the low "neuraminic acid/water-soluble hexose" ratio (Table 1). Calculating this ratio from the figures presented in these cases, one can conclude that only 20% to 30% of the total water-soluble glycolipids are present as gangliosides, the rest being made up by polycerebrosides.

The elevated levels of water-soluble glycolipids, with a relative increase in ganglioside versus nonneuraminic acid containing polycerebrosides observed in the spleen from Niemann-Pick cases, present a rather different problem. An increase in gangliosides in the brain in Niemann-Pick cases had previously been reported by Klenk,[1,2] and a ganglioside had been found as a constant feature enriching the polysaccharide storage substance in gargoylism.[5] Similarly, Diezel,[18] in a thorough histochemical study, has concluded that there is a considerable amount of ganglioside or ganglioside-like material stored together with sphingomyelin in Niemann-Pick cells. It is therefore tempting to consider the accumulation of water-soluble glycolipids as secondary to the primary process that leads to deposition of sphingomyelin in large amounts, with a proportionate decrease in the lecithin and cephalin fractions of the phosphatides. Such a propensity may be due to the favorable spatial lipophilic polarity that sphingomyelin presents when bound to cytoplasmic protein in a specific way, as appears probable in Niemann-Pick's disease.

The in vitro experiments with the Gaucher lipoprotein and lecithin solutions present in essence a model system in which the secondary binding of phosphatides by the GLP is convincingly demonstrated. The fact that the number of residual organic anion-binding sites decrease with increasing amounts of lecithin present (decrease in amount of methyl orange bound) suggests that the nature of the phospholipid binding to the GLP is an electrostatic one. The observation, repeatedly made, of an increase in residual organic anion binding after very small amounts of lecithin are bound by the GLP (in comparison with the original GLP) points to the possible activation of Klotz's "accessory binding sites" so that previously weak binding sites become strengthened by the contribution of hydrogen bonds and van der Waals forces due to the phospholipid. This explanation is strengthened by the data on the release of methyl orange bound by the GLP-lecithin complex through competition with PABA or PASA (Table 2, Columns 2, 3). It is evident that although the amount of methyl orange bound decreases with increasing lipid concentration in the GLP-lecithin complex, that amount of methyl orange which is bound is less easily released (or replaced) by competing carboxyl groups of PABA and PASA. This is especially noteworthy in view of the much smaller molecular size of PABA and PASA in comparison with methyl orange, so that failure to release or replace the other is not easily explained on grounds of the steric hindrance offered by the presence of lecithin to the access of PABA ions to sites previously occupied by methyl orange. Column 4 in Table 2 represents those sites of binding for methyl orange which are not affected by PABA. It is clear, however, that the lecithin already attached to the GLP cannot be replaced by PABA or PASA, since the carboxyl competition would be ineffective against the stronger binding of the phosphoric acid group of lecithin.

Whether or not the nonspecific increase of phospholipids in Gaucher's disease occurs

as a direct result of the increased organic anion avidity of the kerasin-protein complex that represents the Gaucher lipoprotein cannot be answered with certainty. It appears fair to say that the in vitro data support the possibility that this may be the mechanism involved. If this should prove true, the need to postulate multiple intracellular enzymatic defects to explain the increase in phosphatides, cholesterol, and gangliosides in addition to cerebrosides is eliminated, and the original biochemical anomaly can still be traced to the abnormal kerasin-protein binding, with all other lipid changes being secondarily induced by this primary defect.

In Niemann-Pick's disease a somewhat different picture emerges. There is an absolute as well as relative increase in sphingomyelin with respect to other lipids, including lecithins and cephalins. The deposition of sphingomyelin does not appear to favor a significant secondary rise in other phosphatide levels. It does appear, however, to favor the increase of water-soluble glycolipids. It is unfortunate that our inability to produce stable and relatively homogeneous sphingomyelin sols made it impossible to test this hypothesis in vitro in a protein-sphingomyelin-polycerebroside (or ganglioside) system.

Summary

The composition of surgically removed spleens from eight patients with Gaucher's disease and three patients with Niemann-Pick's disease were studied with respect to total lipid, total phosphatides, sphingomyelins, cerebrosides, water-soluble glycolipids (gangliosides and polycerebrosides), and cholesterol. In both diseases an increase in all lipid fractions, together with the characteristic increases in cerebrosides and sphingomyelins, was demonstrated. In two cases of the infantile type of Gaucher's disease the increase in water-soluble glycolipids was found to be much in excess of that of cerebrosides. It is suggested that in such cases, unless cerebrosides are isolated in pure form first, analysis of tissue by previous methods is bound to give erroneous "glucocerebroside" values because of the high glucose content of water-soluble glycolipids. In Niemann-Pick's disease the increase in sphingomyelin was accompanied by an increase in water-soluble glycolipids, with a relatively minor increase in lecithins and cephalins. In both diseases an increase in cholesterol was noted. Dialysis equilibrium experiments, designed to measure residual organic anion-binding capacity of the lipoprotein of Gaucher's disease, showed that lecithin sols, artificially produced, are readily bound by the natural kerasin-protein complex in vitro. It is suggested that the increase in nonspecific lipid fractions in primary lipoid storage diseases is secondary to the original deposition and binding by cytoplasmic proteins of kerasin and sphingomyelin, respectively. The relative proportions in which phosphatides, cholesterol, and water-soluble glycolipids are secondarily increased is considered to depend on the nature of the spatial arrangement of lipophilic residues and the residual organic anion-binding sites resulting from the lipid-protein complexes originally formed with cerebrosides and sphingomyelin.

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News and Comment

ANNOUNCEMENTS

Course in Experimental Immunohematology.—A course in experimental immunohematology is being offered at the Mount Sinai Medical Research Foundation, Chicago, during the month of May, 1958. The course is intended to provide training in methodology of clinical and experimental immunohematology, including hemolytic anemia, infectious mononucleosis, and isosensitization. A maximum of four candidates will be accepted for the four-week course. There will be no tuition fee.

Preference will be given to pathologists with interest in experimental work. Applications are to be sent to Dr. Israel Davidsohn, Director of Research, Mount Sinai Medical Research Foundation, 2755 W. 15th St., Chicago 8, and should be accompanied by a curriculum vitae and two professional references.

Deadline for the application is March 15, 1958, and notifications of final disposition will be sent out on or about April 1, 1958.

This course is supported (in part) by Grant H-3413 from the National Institutes of Health, United States Public Health Service.

Glomerular Lesions Produced in the Rabbit by Prednisone and Prednisolone

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After administration of cortisone to rabbits for short periods, kidney lesions with some morphologic features of diabetic glomerulosclerosis[1] have been described by Rich and his co-workers,[2] by Bloodworth and Hamwi,[3,4] and by Wilens and Stumpf.[5] The histologic changes consist of striking glomerular capillary dilatation and nodular glomerular lesions which stain positively with periodic acid-Schiff (PAS) stain, fat stains, and fibrin stains. Deeply staining amorphous casts are frequently noted in various portions of the renal tubular system. In the course of studies on the nature and significance of these lesions the effects of steroids other than cortisone on rabbit kidney morphology were investigated. This report deals with experiments carried out using the Δ^1 synthetic steroids, prednisone and prednisolone.* These substances are reported to have greater anti-inflammatory activity than cortisone, while exerting negligible effects on carbohydrate or electrolyte balance.[6]

Material and Methods

Twenty-four male New Zealand white rabbits were employed. Six were normal animals, and ten had been made diabetic six weeks previously with a single intravenous dose of alloxan monohydrate. The left kidney of each of these 16 animals was removed with use of pentobarbital (Nembutal) and ether anaesthesia to serve as control material. The animals were maintained on Sherwood feed unsupplemented by cyanocobalamin (vitamin B_{12}) or chlortetracycline (Aureomycin) and were provided with 1% sodium chloride solution as drinking water. The diabetic group received 10 mg. of prednisone suspension intraperitoneally each day and the nondiabetic group, 5 mg. Wherever the condition of the animal permitted the steroid was continued for a period of 21 days.

A further group, of eight rabbits, received a daily intraperitoneal injection of 5 mg. of prednisolone suspension. After 21 days the left kidney was removed, and the animals continued to be given 15 mg. of prednisone intraperitoneally each day for a further 21 days. These animals were maintained on a similar feed but were provided with ordinary tap water ad libitum.

In all groups, body weight, nonfasting blood glucose, and 24-hour urine glucose were measured twice weekly. Blood pressure was measured in the prednisone-treated nondiabetic group by auscultation over the aorta below a pediatric sphygmomanometer cuff.

Kidney slices were fixed in formol and Zenker-formol, sectioned at 2.5μ, and stained with hematoxylin, phloxine, and saffron.[7] Additional sections were stained with PAS, oil red O, Masson trichrome, and phosphotungstic acid and hematoxylin.

Results

The blood sugar level in the diabetic rabbits ranged between 200 and 570 mg. per 100 cc. and was unaffected by prednisone administration. The blood sugar level of the nondiabetic animals receiving either prednisone or prednisolone remained within normal limits. In all cases the body weight remained essentially unchanged throughout the experiment. The nondiabetic rabbits given prednisone were found to have a mean maximum blood pressure increase of 31 mm. of mercury systolic and 33 mm. of mercury diastolic. All animals were noted to excrete

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* The prednisone and prednisolone used in these studies were provided by J. W. Brisick, of the Schering Corporation Limited.

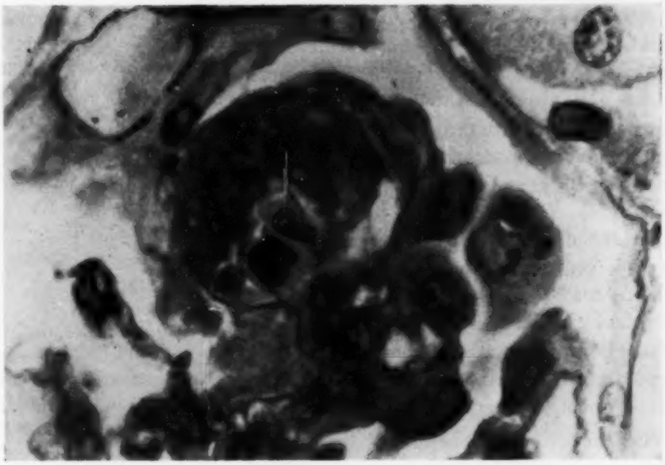

Fig. 1.—Glomerulus of cortisone-treated rabbit. Illustrates dilated capillary containing hyaline material and fatty vacuoles. PAS; reduced 19% from mag. × 1400.

amber urine containing a large quantity of sediment. The urine was not found to react with benzidine.

The kidneys removed at time of autopsy presented a petechial mottling on the capsular and cut surfaces of the cortex; no other gross alteration was observed. All animals which had received prednisone and/or prednisolone for 21 days showed characteristic alterations indistinguishable from those observed with cortisone by ourselves and others [2-5] (Figs. 1 and 2). Kidneys from the diabetic group of animals presented, in addition, evidence of acute pyelonephritis and tubular-cell calcium accumulation.

While the effects of prednisolone and cortisone were similar, those of prednisone at the dosages employed were more numerous and severe. Preexisting diabetes and the presence of hypertension had no significant influence on the severity or frequency of lesions.

Comment and Conclusions

Our own histologic studies have led us to agree with those authors [2,4,8] who find that the cortisone-induced lesions of the rabbit kidney resemble those of human diabetic glomerulosclerosis. A relationship is sug-

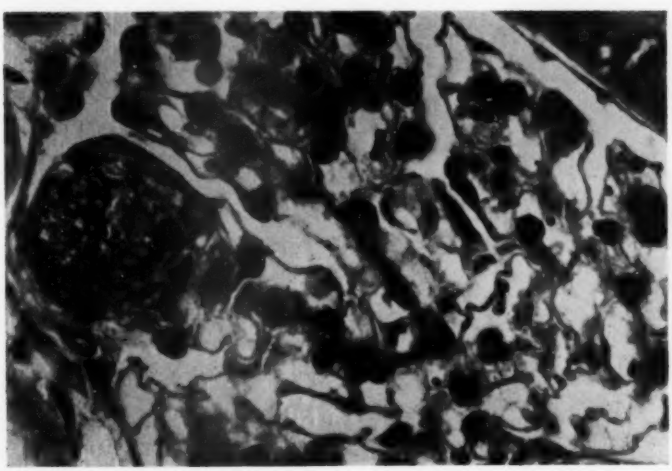

Fig. 2.—Glomerulus of prednisone-treated rabbit. Illustrates dilated capillary containing a fibrillar material in meshes of which is a homogenous substance staining pale blue with Masson's trichrome; reduced 19% from mag. × 750.

gested too by recent studies of Sommers and Haley,[9] who have demonstrated in the glomeruli and arterioles of nondiabetic humans and animals treated with cortisone the presence of an abnormal substance identical in ultraviolet absorptive properties with the nodules of human intercapillary glomerulosclerosis. The present study demonstrates that the Δ^1 substituted steroids, prednisone and prednisolone, can produce similar kidney damage to that observed in our cortisone studies. It is particularly significant that these lesions occur in nondiabetic animals and are not influenced by alloxan diabetes or experimental hypertension.

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Experimental Tuberculous Meningitis in Rabbits

1. Results of Treatment with Antituberculous Drugs Separately and in Combination with Cortisone

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The clinical indications and contraindications for adrenocortical therapy in tuberculosis have been the subject of considerable controversy in the last few years. It had been previously concluded that corticotropin (ACTH) and cortisone are contraindicated in tuberculosis because of their adverse effect on existing tuberculous lesions [1] and because of the flare-up of tuberculosis [2] in patients treated with these hormones for systemic conditions. Subsequent clinical observations have indicated, however, that tuberculosis need not constitute a contraindication provided intensive antituberculous therapy is given at the same time.[3] Furthermore, in view of their anti-inflammatory and antiallergic actions these hormones have been introduced as adjuvants in the treatment of tuberculosis, and satisfactory clinical results have been reported in certain forms of the disease,[4-6] including tuberculous meningitis.[7-10]

There are a number of investigations on the effect of combined antituberculous and adrenocortical therapy on experimental tuberculosis in animals,[4] but no such studies have been reported in experimental tuberculous meningitis.

In order to obtain more information on the results of the combined therapy, the effects of streptomycin and isoniazid administered separately as well as in combination with cortisone were determined on experimental tuberculous meningitis and on the formation of pia-arachnoid adhesions in the course of its healing.

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Department of Nervous Diseases, Laboratory of Experimental Neurology, and Departments of Pathology and Clinical Microbiology, Hadassah University Hospital and the Hebrew University Hadassah Medical School.

Material and Methods

Rabbits weighing approximately 2 kg. were used. The meningitis was experimentally induced in 66 animals by the following method: with use of light ether anesthesia approximately 100,000 tubercle bacilli of Mycobacterium tuberculosis var. bovis * suspended in 0.2 ml. of saline were introduced under sterile conditions into the cisterna magna after the withdrawal of an equal amount of cerebrospinal fluid. A culture sensitive to 0.5μg. per milliliter of streptomycin and isoniazid, respectively, was used. Treatment, unless otherwise stated, was started 15 days after the infection. All the drugs were administered intramuscularly daily.

The lesions observed in four untreated controls between the 15th and 17th days after infection formed the basis for histological evaluation of the effect of the various treatments on the experimental infection.

1. *Streptomycin.*—Four animals received 50 mg.; seven animals, 100 mg., and five animals, 200 mg. of streptomycin.

2. *Streptomycin and Isoniazid.*—Five animals received 200 mg. of streptomycin for two weeks and subsequently 50 mg. of isoniazid until the end of the experiment.

3. *Cortisone.*—Two animals received 15 mg. of cortisone.

4. *Streptomycin Plus Cortisone.*—Three animals received 50 mg. of streptomycin and 15 mg. of cortisone; three animals, 100 mg. of streptomycin and 15 mg. of cortisone, and one animal, 200 mg. of streptomycin and 15 mg. of cortisone.

5. *Streptomycin and Isoniazid Plus Cortisone.*—Seven animals received 200 mg. of streptomycin for two weeks and later 50 mg. of isoniazid plus 15 mg. of cortisone until the end of the experiment.

6. *Isoniazid.*—Fourteen animals were treated with 50 mg. of isoniazid daily in the following manner:

* The strain was supplied by the Veterinary Institute of the Ministry of Agriculture.

In 4 of them the treatment was started one day, and in the other 10, fifteen days after the inoculation.

7. *Isoniazid Plus Cortisone.*—Eleven animals were treated with 50 mg. of isoniazid and 15 mg. of cortisone daily.

Six normal rabbits received 100 mg. of streptomycin twice daily for four days, and the streptomycin level in their cerebrospinal fluid was determined.

Results

All but four rabbits which received streptomycin, whether alone or in combination with cortisone, died within the first five weeks after the infection. One animal, however, which received 50 mg. of streptomycin, survived 39 days; another, which received 100 mg. of streptomycin, survived for 45 days, and a third, which received 200 mg. of streptomycin, was well when killed 55 days after the infection. One animal which received 200 mg. of streptomycin plus cortisone died on the 55th day. Animals which received streptomycin and subsequently isoniazid, with or without cortisone, survived for longer periods, namely, from five to eight weeks. Rabbits which received cortisone alone died at the end of one month.

Weakness and subsequent paralysis of the hindlegs appeared a week or two before death, the paralysis of the forelegs appearing later.

Of the 14 animals treated with isoniazid alone, 10 were killed between the 37th and 193d days and 4 died between the 30th and 60th days after the intracisternal inoculation with tubercle bacilli. Of the 10 animals treated with isoniazid plus cortisone, 4 were killed between the 30th and 90th days and 6 died between the 37th and 128th days after the cisternal inoculation.

Macroscopic Findings.—In all animals except those which either received isoniazid immediately after the infection or were treated with isoniazid plus cortisone the gross picture was essentially one of basal meningitis or arachnoiditis. In the nontreated animals many tubercles about 1 mm. in diameter were spread over a thickened, opaque, and friable pia-arachnoid membrane. In two of the animals treated with 50 mg. of streptomycin the picture was similar, except that there was also a diffuse subarachnoid hemorrhage. In the other streptomycin-treated animals, the leptomeninges showed thickening of a various degree, while the tubercles lay at a greater distance from each other and were smaller and harder. The same picture was found in the group treated with either streptomycin and isoniazid or streptomycin and isoniazid plus cortisone. Animals treated with cortisone alone showed a very considerable thickening with friability of the leptomeninges. The tubercles were very large, some of them more than 2 mm. in diameter, with soft and umbilicated centers.

A similar picture but of less degree was observed in the rabbits treated with streptomycin plus cortisone. The animals treated with isoniazid alone showed a slight leptomeningeal thickening, mainly along the Sylvian fissures. In many areas of the basal cerebral surfaces the leptomeninges looked normal. The brains of animals treated with isoniazid immediately after infection or with isoniazid plus cortisone showed nothing abnormal.

On coronal sections, hydrocephalus of a mild to moderate degree was found in all experimental animals except those treated with isoniazid immediately after infection and those treated with isoniazid plus cortisone.

Histological Findings.—Nontreated Animals: Between the 15th and 17th days the leptomeninges of the nontreated animals were found to be infiltrated by small masses of epithelioid cells in close apposition to each other, as well as by many lymphocytes, plasmacytes, and granulocytes. Among some of these epithelioid-cell collections, there was necrosis of the inflammatory cellular exudate, as identified by foci of karyorrhexic nuclear debris mixed with granulocytes. The walls of some pial veins and arteries were infiltrated by many granulocytes, and here,

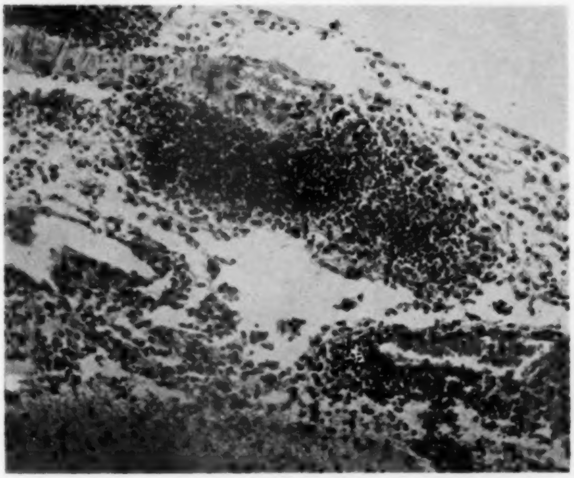

Fig. 1.—The tuberculous process in the leptomeninges on the 15th day, just before starting treatment. Hematoxylin and eosin; × 110.

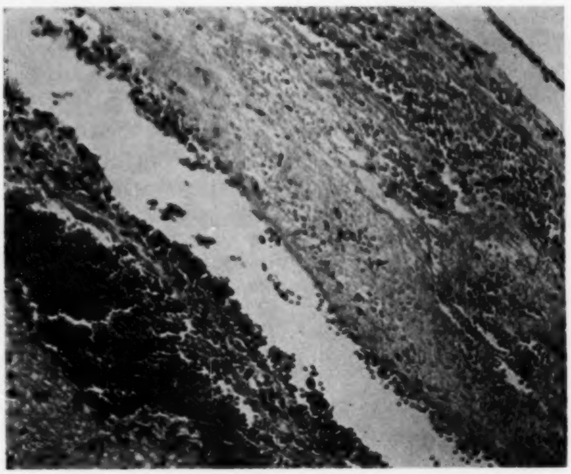

Fig. 2.—Massive fresh subarachnoid hemorrhage due to necrotizing angiitis of the tuberculous leptomeninges. Hematoxylin and eosin; × 150.

too, there was evidence of focal necrosis (acute necrotizing angiitis) (Fig. 1). Many acid-fast bacilli were found when sections were stained by the Ziehl-Neelsen method.

Streptomycin-Treated Animals: Two of the four animals treated with 50 mg. of streptomycin daily and which died on the 25th and 27th days, respectively, revealed a diffuse exudative tuberculous meningitis with a marked distention and obstruction of the subarachnoid space. There was also a fresh massive subarachnoid hemorrhage (Fig. 2) due to acute necrotizing angiitis of pial vessels. In other places, however, the acute exudative angiitis was replaced by an epithelioid granulomatous process (Fig. 3), which in some vessels involved the whole thickness of the wall and caused stenosis and even obliteration of the vascular lumen.

On the other hand, in the two other animals which were also treated with 50 mg. of streptomycin and survived for 33 and 39 days, respectively, as well as in the animals which received 100 mg. of streptomycin and had a maximum survival of 45 days, histological examination showed no necrotizing but only epithelioid granulomatous angiitis and periangiitis of pia-arachnoid vessels.

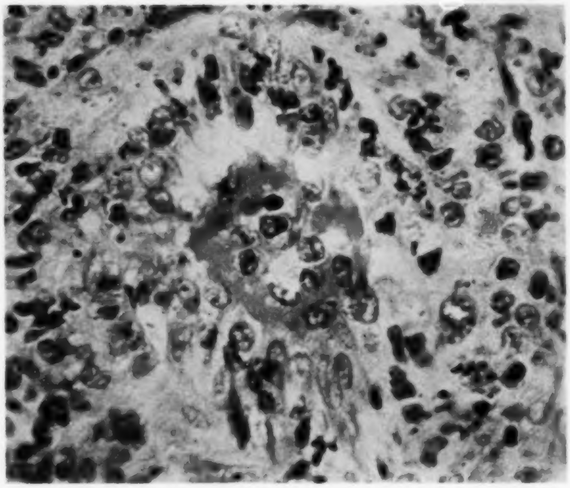

Fig. 3.—Epithelioid granulomatous (tuberculous) panvasculitis with extreme stenosis of lumen. Here and there is found residue of the acute necrotizing inflammation of the vascular wall, in the form of nuclear debris. Hematoxylin and eosin; × 500.

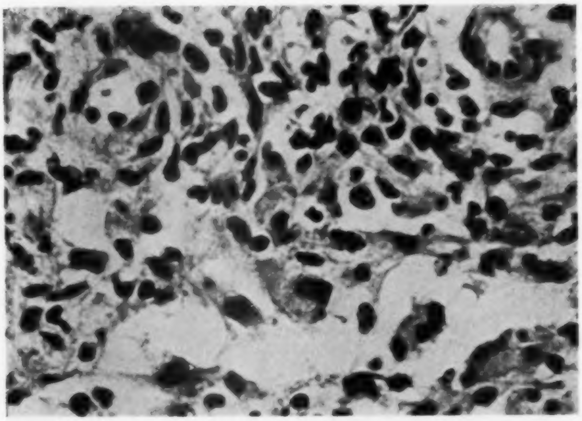

Fig. 4.—Transformation of epithelioid cells into fibroblasts arranged in a reticular manner. Hematoxylin and eosin; × 500.

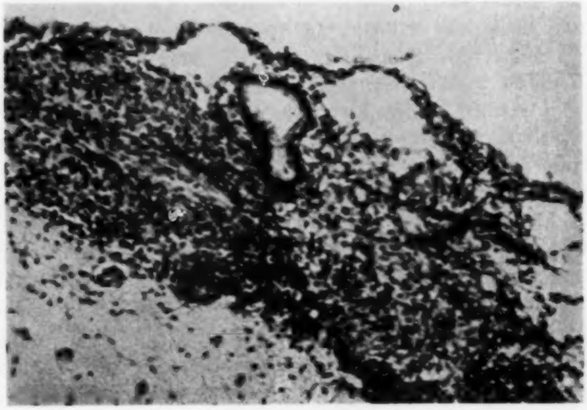

Fig. 5.—Beginning of deposition of fibrils within the organizing tuberculous process of the leptomeninges. Laidlaw's reticulin stain; × 150.

Fig. 6.—Fibrous thickening of leptomeninges in a rabbit treated with 200 mg. of streptomycin for 55 days. Hematoxylin and eosin; × 500.

There was also evidence of a chronologically advancing transformation of epithelioid cells into fibroblasts (Fig. 4), with deposition of eosinophilic fibrillary intercellular substance staining positive for collagen, and presence of a reticulin network (Fig. 5) which was the more intricate the longer an animal had survived. Tubercle bacilli were identified in the brains of these animals. In the animal which received 200 mg. of streptomycin and was killed on the 55th day, the histological examination showed fibrous thickening of the leptomeninges (Fig. 6). There were also focal obliterations of the subarachnoid space by masses of fibrosed and shrunken epithelioid granulomata traversed in all directions by a rich reticulin network (Fig. 7), except at their centers, where caseation was still present.

Streptomycin- and Isoniazid-Treated Animals: In animals examined about the 40th, 45th, and 55th days after inoculation, the pathological picture was similar to that observed, at the respective stages, in animals treated with the higher doses of streptomycin alone. The impression was, however, that in the latter the fibrosis was more pronounced.

Cortisone-Treated Animals: In these two animals, both of which died at the end of the first month after inoculation, there was found massive caseative tuberculous leptomeningitis, with tremendous distention of the subarachnoid space (Fig. 8) of a degree observed in none of the other experimental animals.

Streptomycin-Plus-Cortisone-Treated Animals: These animals showed at the end of the first week, after initiation of treatment, widespread and extensive caseative

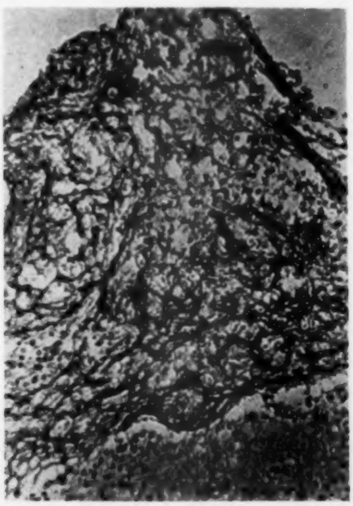

Fig. 7.—Obstruction of subarachnoid space by the organized tuberculous leptomeninges. A rich reticulin network is seen traversing the subarachnoid space and causing pia-arachnoid adhesions. Laidlaw's reticulin stain; × 85.

Fig. 8.—Massive caseative tuberculous meningitis causing subarachnoid obstruction in a rabbit treated with cortisone alone. Hematoxylin and eosin; × 110.

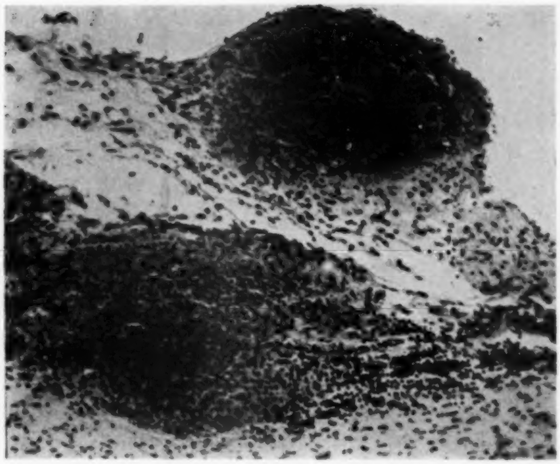

Fig. 9.—Widespread and extensive caseative tuberculous leptomeningitis in a rabbit treated for one week with streptomycin plus cortisone. Hematoxylin and eosin; × 110.

tuberculous meningitis (Fig. 9). Around pial veins and arteries as well as within their walls numerous large epithelioid granulomata were seen, all of them extensively caseated and many of them fused together into large tuberculomata, blocking completely the subarachnoid space. No acute or necrotizing angiitis or massive subarachnoid hemorrhage were observed. In addition to this, animals of this group which survived for more than four weeks (two weeks' treatment) showed a peculiar rarefaction of the granulomata, with hydropic degeneration and karyorrhexis of many epithelioid cells. At the periphery of the granulomata there was observed a small degree of fibroblastic transformation of epithelioid cells. At 40 days there was still no substantial fibrosis of the granulomata, a slight deposition of collagen, and very few reticulin fibers (Fig. 10) between the epithelioid cells, the latter retaining their characteristic morphology. These still formed tubercles with caseous centers, which, although small in size, made easy the histological diagnosis of tuberculous meningitis.

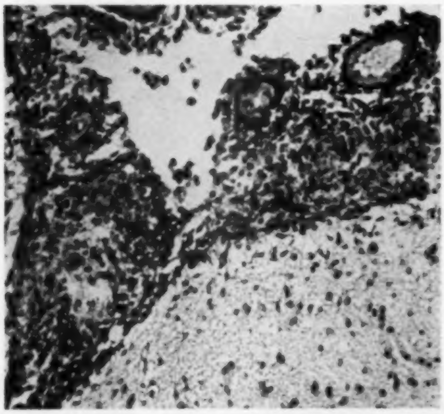

Fig. 10.—Rabbit treated with streptomycin plus cortisone. At 24 days after initiation of treatment there is still no substantial fibrotic transformation of the epithelioid granulomata and almost no deposition of reticulin fibrils. Laidlaw's reticulin stain; × 150.

Streptomycin- and Isoniazid-Plus-Cortisone-Treated Animals: These animals showed changes similar to those observed at same stages in the streptomycin-and-isoniazid-treated group, but there was less deposition of intercellular substance.

In all animals various degrees of involvement of the brain tissue by the tuberculous process were found. In most animals tubercle bacilli were identified by the Ziehl-Neelsen stain.

Isoniazid-Treated Animals: The leptomeninges of these animals treated immediately after the infection and killed two months later revealed a normal appearance but for a few lymphocytes infiltrating them. Animals treated with isoniazid after an interval of two weeks showed complete heal-

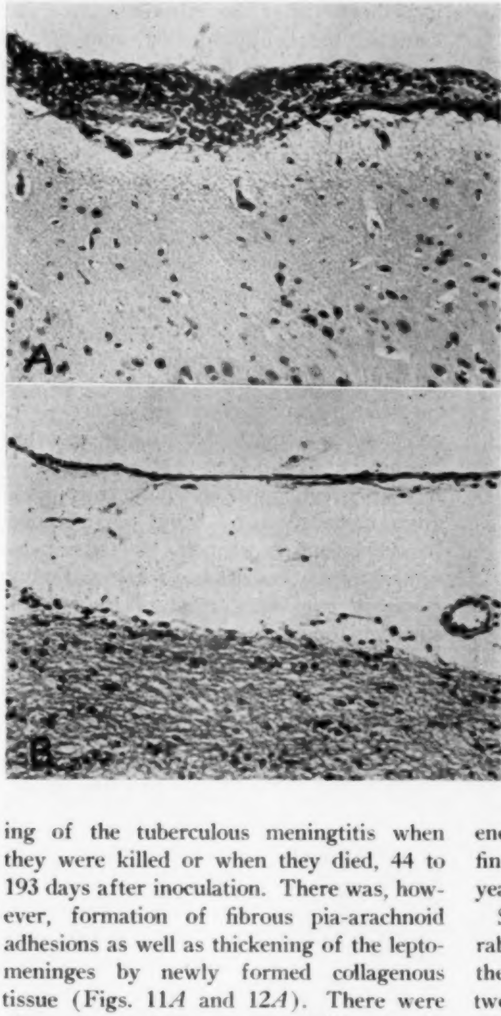

Fig. 11.—*A*, rabbit treated with isoniazid for 44 days. Leptomeninges show fibrous thickening and infiltration by nonspecific chronic inflammatory cells (lymphocytes). Subarachnoid space is obliterated. Hematoxylin and eosin; × 150. *B*, rabbit treated with isoniazid plus cortisone for 44 days. Leptomeninges are normal, subarachnoid space is patent. Hematoxylin and eosin; × 150.

ing of the tuberculous meningtitis when they were killed or when they died, 44 to 193 days after inoculation. There was, however, formation of fibrous pia-arachnoid adhesions as well as thickening of the leptomeninges by newly formed collagenous tissue (Figs. 11*A* and 12*A*). There were also minute foci of nonspecific chronic meningoencephalitis.

Isoniazid-Plus-Cortisone-Treated Animals: The leptomeninges of animals in which treatment with isoniazid plus cortisone was also started 15 days after the infection showed neither thickening nor adhesions (Figs. 11*B* and 12*B*) when they were killed or when they died, 44 to 128 days after inoculation.

In the isoniazid, and to less degree in the isoniazid-plus-cortisone-treated animals, occasional foci of nonspecific nonpurulent encephalitis were found. This was also the finding in the animal which survived for one year.

Streptomycin Determinations: In all six rabbits the concentration of streptomycin in the cerebrospinal fluid was found to be between 0.16 and slightly less than 1 unit per milliliter.

Comment

The present investigation demonstrated that the systemic administration of streptomycin, even in high doses, was unable to stop the progress of tuberculous meningitis in most of the animals. This effect in rabbits is very similar to that in the human disease [11] and explains the failure of this therapy to save many patients. Though there was a certain amount of healing with use of streptomycin treatment, the experimental animals died of meningeal tuberculosis, which

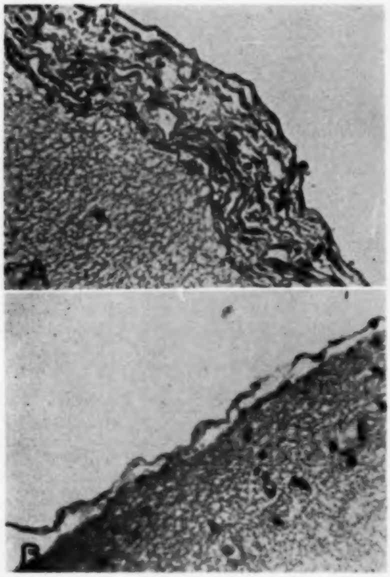

Fig. 12.—*A*, rabbit treated with isoniazid for 76 days. Fibrous thickening of leptomeninges with obliteration of subarachnoid space. Hematoxylin and eosin; × 350. *B*, rabbit treated with isoniazid plus cortisone for 76 days. Leptomeninges are normal, subarachnoid space is patent. Hematoxylin and eosin; × 350.

caused massive subarachnoid blocks and hydrocephalus. Some animals had subarachnoid hemorrhages, which were probably due to acute necrotizing angiitis. In high doses streptomycin caused a progressive fibrosis in the meninges, with deposition of reticulin and collagen. There remained, however, tuberculous foci in the obliterated subarachnoid space. The substitution of isoniazid for streptomycin one month after the infection, at the time when neurological signs had already appeared in some rabbits, did not prevent the development of the tuberculous process but it prolonged its course.

Isoniazid therapy, on the other hand, also started 15 days after the infection, was found to be an effective antituberculous drug. The healing, however, was accompanied by the formation of fibrous arachnoiditis and by a mild hydrocephalus. No fibrous adhesions were found in animals in which isoniazid treatment was started immediately after the infection. This fact confirms the ability of early-administered isoniazid to prevent the development of experimental tuberculosis.[12]

Only seldom is tuberculous meningitis diagnosed in man at its very beginning, and usually a certain period of time will elapse before the specific therapy is started. Therefore, treatment in our experiments was started on the 15th day after infection. Histological examinations showed a well-developed tuberculous meningitis already present at that time.

Only a few studies on the effect of antituberculous drugs on experimental tuberculous meningitis have been reported. Steenken et al.[13] described the beneficial effect of streptomycin in guinea pigs when given immediately following the infection. In subsequent experiments [14] streptomycin administration was delayed for 16 days, but, unlike in our study, most of the animals survived for more than 6 months and died with active tuberculous meningitis and hydrocephalus only after the therapy had been discontinued. In experiments reported by Stevens et al.,[15] guinea pigs which were treated with streptomycin from the 14th day after intrathecal inoculation survived for more than six months, when hydrocephalus of a mild degree and fibrous thickening of the meninges were found. Some meningeal fibrosis, however, was already present in animals which died during the second week of therapy. It is possible that the strains of M. tuberculosis used in the above-mentioned experiments were more susceptible to streptomycin, or less prone to develop resistance to this drug, than the strain we used. Another possibility would be that streptomycin is concentrated in the cerebrospinal fluid of guinea pigs at higher levels than in rabbits.

Fust and Studer,[16] Musotto and Lauro,[17] and Levaditi et al.[18,19] reported the beneficial effect of isoniazid on experimental tuberculous meningitis provided treatment was started simultaneously with the infection. Isoniazid was less effective [17] or in-

effective [16] if treatment was delayed. The death of a number of rabbits in these experiments could not be attributed to the experimental infection [16,17] but rather to the toxicity of isoniazid.[16] Wolinsky et al.,[14] in evaluating four modes of treatment (iproniazid, isoniazid, streptomycin or isoniazid and streptomycin) on experimental tuberculous meningitis in guinea pigs, found no superiority of one over the others. Irrespective of whether the isoniazid treatment was started immediately or delayed for 16 days, most of the animals remained alive for more than 6 months. When therapy was discontinued, most animals died within the next few months with tuberculosis of the meninges and the brain and hydrocephalus. Similar relapses were observed in the experiments of Musotto and Lauro,[17] who treated their rabbits with isoniazid for shorter periods of time. In our study the animals were treated until the end of the experiment, namely, up to six months. The occasional focal inflammation found in their brains may indicate that the infection had not yet been completely irradicated and that a longer period of therapy is necessary.

The effect of systematically administered antituberculous drugs on experimental tuberculous meningitis is to a great degree dependent on their ability to cross the blood-cerebrospinal fluid barrier. In this respect not only are there differences among the various substances, but the same drug may have a different permeability in different species.[20] As judged from our studies on the effect of streptomycin on the experimental infection, and from its determinations in the cerebrospinal fluid, streptomycin in rabbits achieves a low concentration in the cerebrospinal fluid. Isoniazid, on the other hand, seems to pass into the cerebrospinal fluid easily and produces healing of the tuberculous process. Similar findings with streptomycin [21,22] and isoniazid [23] in man have been reported. As noted in the review of literature and in our experiments, there is a difference between the therapeutic effect of streptomycin on experimental tuberculous meningitis in rabbits and that in guinea pigs. The time of initiation of therapy as well as the quantities of the tubercle bacilli inoculated were similar in the various studies mentioned. In guinea pigs doses of 3[14] and 4[15] mg. per 100 gm. of body weight were able to suppress the tuberculous process in the meninges, while doses up to 10 mg. per 100 gm. of body weight in rabbits caused, in our experiments, only some prolongation in the course of the disease but could not save most of the animals. This may be due to the low levels (0.16 to less than 1 unit per milliliter) at which streptomycin is concentrated in the cerebrospinal fluid of rabbits, even when administered in large doses (100 mg. per kilogram of weight daily).

The deleterious effect of cortisone alone on experimental infections including tuberculosis has been described in a number of reports.[24] Among other reasons, this is due to the depressing effect of cortisone on inflammatory reactions, granulation-tissue formation, and macrophage activity.[25,26] Morgan et al,[27] who investigated the effect of cortisone and streptomycin on experimentally induced pulmonary tuberculosis in rabbits, found that, while large doses of cortisone enhanced the disease in spite of the action of streptomycin, small doses of cortisone did not affect its therapeutic action. Furthermore, Even et al.[4] reported that the hormone together with streptomycin had apparently a greater therapeutic effect than streptomycin alone. The permeability of the blood-cerebrospinal fluid barrier to cortisone is another factor upon which the effect of the combined therapy in the experimental animals depends. Chemical determinations of corticoids in the cerbrospinal fluid in humans [28,29] and the influence of cortisone, administered parenterally, on the meningeal inflammation in humans [9,10] and rabbits [30] suggest that they enter the cerebrospinal fluid in effective concentrations. In animals which received cortisone alone, there was a strikingly enhancing effect on the meningeal tuberculous process leading

to massive caseation. This may explain the development of tuberculous meningitis reported in patients who harbored a latent infection and received cortisone for another condition.[31,32] The addition of cortisone to streptomycin—by itself unable to control the disease effectively—had a deleterious effect in our experiments. No necrotizing angiitis, however, and no subarachnoid hemorrhage were observed in this group. The vascular damage, being probably the result of an allergic response to the tuberculoprotein,[33] was reduced due to the antiallergic action of cortisone. In animals treated with isoniazid plus cortisone, the hormone did not interfere with the antituberculous activity of isoniazid. Isoniazid and cortisone therapy was also found to be superior to streptomycin and cortisone therapy in tuberculous peritonitis.[34] Thus the effectivity of the former combination in tuberculous meningitis is not due only to the greater penetration of isoniazid into the cerebrospinal fluid.

No anatomical basis could be found in those animals which died in the isoniazid- and isoniazid-plus-cortisone-treated groups. Their death could be attributed to the toxic action of isoniazid [16,17] and to the emaciating effect of cortisone on rabbits.[30] Therefore, the histological picture, rather than the survival of the animals, should be considered as a criterion of the effectiveness of therapy in these groups.

Before the discovery of streptomycin, tuberculous meningitis was almost inevitably fatal. Even with the introduction of this drug, the mortality was still over 50%.[22,35] One of the main reasons for the failure of streptomycin therapy was its limited ability to penetrate the blood-cerebrospinal fluid barrier in sufficient amount and to check the meningeal infection. Another is the presence of subarachnoid tuberculous exudate and the subsequent formation of fibrous adhesions, which seriously interfere with the normal circulation of the cerebrospinal fluid and produce hydrocephalus.[11,35]

The introduction of isoniazid, which easily enters the cerebrospinal fluid and penetrates the tuberculous exudates, improved the prognosis considerably. The present investigation demonstrates, however, that if the therapy with this drug is started at a time when a considerable subarachnoid exudate has already been established, the latter will undergo progressive organization into connective tissue and produce a fibrous subarachnoid block. In order to prevent the organization of the basal exudates, streptokinase-streptodornase was administered intrathecally. This therapy, however, was discarded because of its being both ineffective and dangerous.[22]

Adrenocortical hormones depress inflammatory processes and connective tissue formation [25] and were therefore introduced as adjuvants in the therapy of tuberculous meningitis. The addition of cortisone and corticotropin to antituberculous drugs as therapy for comatose patients showing signs of subarachnoid block caused regression of the block and a dramatic improvement in the clinical state. There was also a rapid return of the spinal fluid findings to normal and a considerable diminution of the neurological sequelae.[7-10]

In our experiments the administration of isoniazid plus cortisone was started in the presence of a subarachnoid exudative block. As judged from the pathological examinations, cortisone was able to resolve this exudate and thus prevent its transformation into fibrous pia-arachnoid adhesions, such as developed in the animals receiving isoniazid alone. This is probably the mechanism by which adrenocortical therapy exerts its beneficial effect in patients with tuberculous meningitis. The inhibitory action of cortisone on the formation of adhesions in the course of the healing of tuberculous meningitis is in accordance with our previous finding in nonspecific meningitis in cats.[26]

The present investigation confirms experimentally the clinical impression of the beneficial effect of adrenocortical hormones when administered together with effective

antituberculous drugs and their ability to prevent the formation of a fibrous subarachnoid block in tuberculous meningitis.

Summary and Conclusions

Tuberculous meningitis was produced in rabbits by the intracisternal administration of Mycobacterium tuberculosis var. bovis. The results of treatment with antituberculous drugs, separately and in combination with cortisone, were determined.

Most of the streptomycin-treated animals died within five weeks after the induced infection. In the subarachnoid space of these animals various degrees of transformation of the tuberculous exudate into connective tissue were found. A number of animals had necrotizing angiitis with subarachnoid hemorrhage. The substitution of isoniazid for streptomycin prolonged the course but did not cure the tuberculous process.

Cortisone alone had a deleterious effect, resulting in massive caseation. The addition of cortisone to streptomycin had a similar but less pronounced effect, and it inhibited considerably the formation of connective tissue. The later substitution of isoniazid plus cortisone for streptomycin also did not change the course appreciably.

When isoniazid was given immediately after the infection there was complete healing of the tuberculous process. If treatment was delayed for two weeks, the healing was accompanied by the formation of fibrous pia-arachnoid adhesions. Isoniazid plus cortisone therapy started at the same time caused complete healing of the tuberculous meningitis without any residual adhesions.

The literature on the results of treatment of experimental tuberculous meningitis by streptomycin and isoniazid is reviewed, and the importance of the degree of passage of the drugs into the cerebrospinal fluid in different species, emphasized.

The complications arising in the course of healing of tuberculous meningitis are discussed, and the beneficial effect of adrenocortical hormones as adjuvants, mentioned. The present investigation demonstrates that isoniazid therapy leads to healing with formation of fibrous adhesions, if administered at a time when tuberculous subarachnoid exudate is already present. If administered together with cortisone, the formation of these adhesions was prevented.

The present investigation offers experimental evidence for the beneficial effect of the combined isoniazid and adrenocortical therapy in tuberculous meningitis.

Hebrew University-Hadassah Medical School (Dr. Behar).

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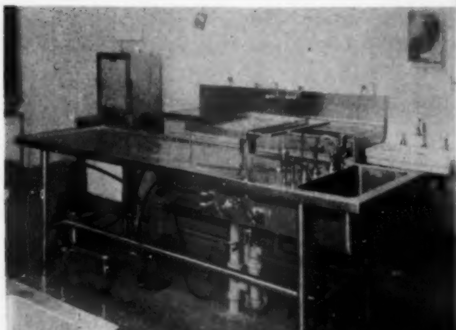

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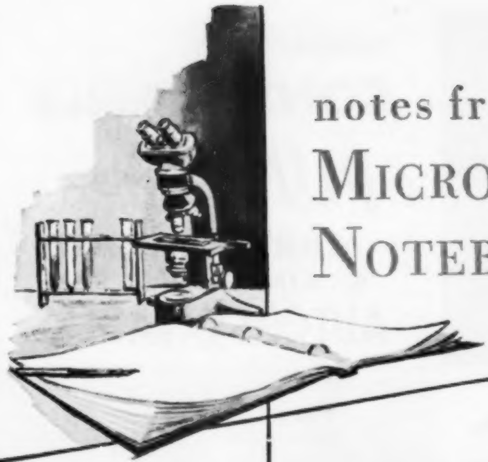

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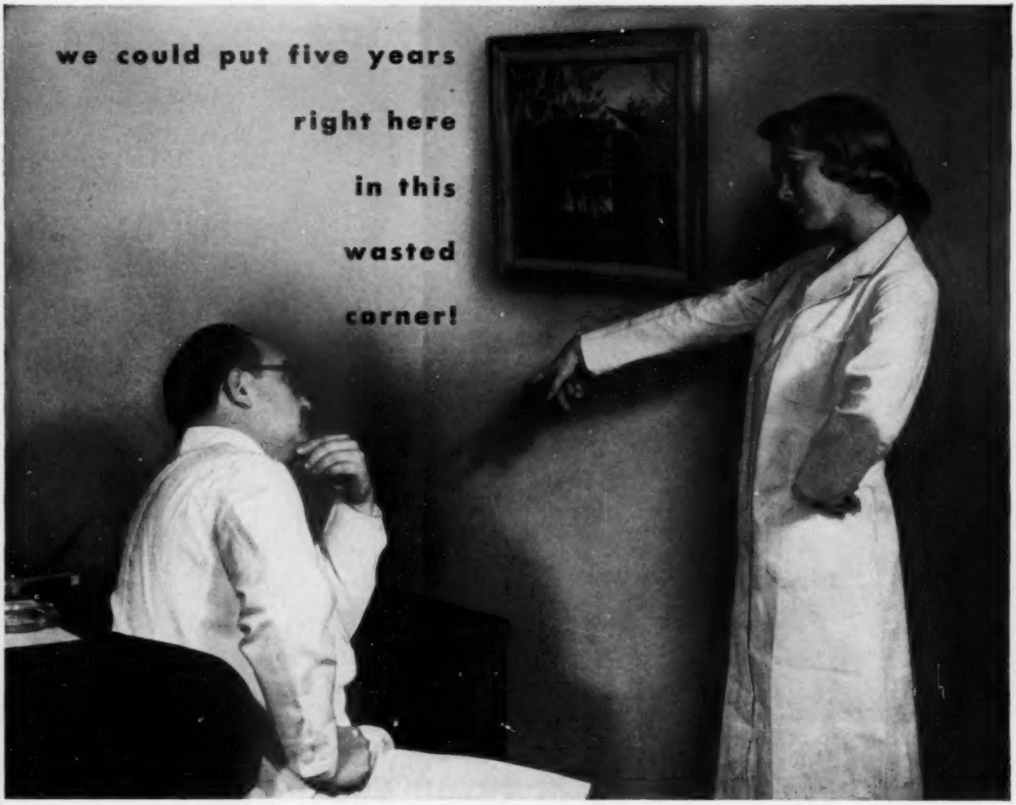

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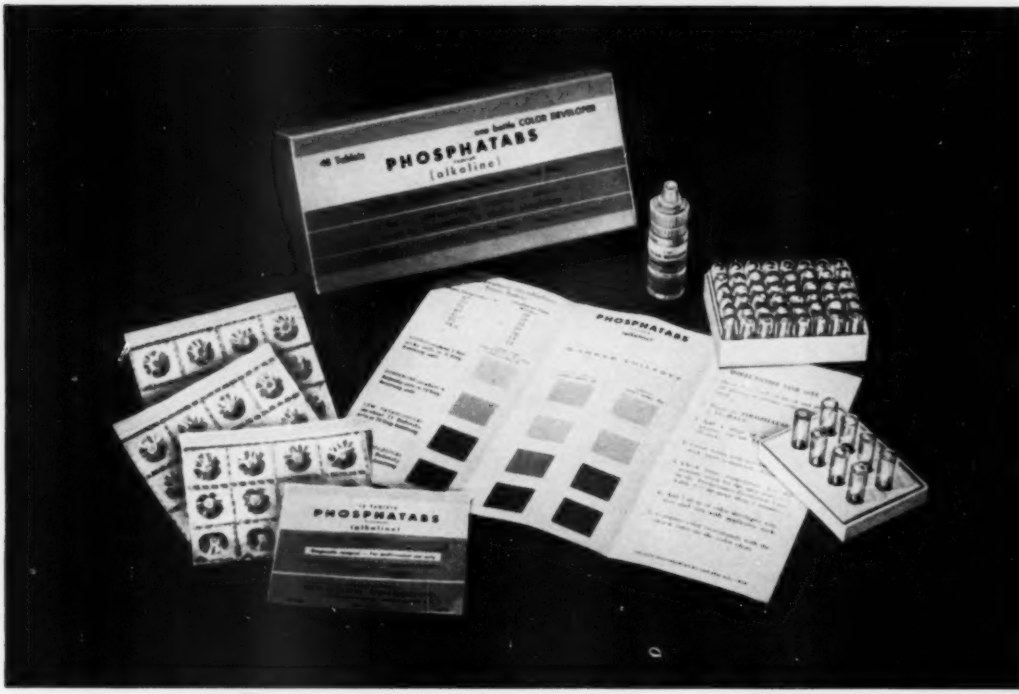

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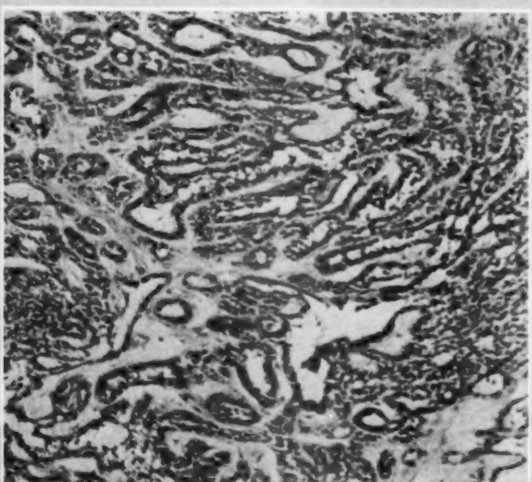

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Photomicrograph (x 90) *(left)* showing the alveolar glandular pattern so characteristic of certain fields of an adamantinoma.

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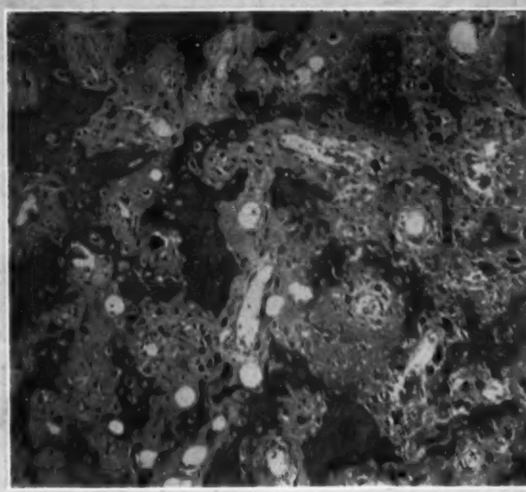

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Radiograph *(upper left)* of a highly ossifying osteogenic sarcoma in the upper part of the shaft of tibia of a boy of 10 years.

Photograph *(upper right)* of longitudinal section of tibia shown in radiograph above. Part of the cortex and epiphyseal plate have been destroyed, but the epiphysis is still not invaded.

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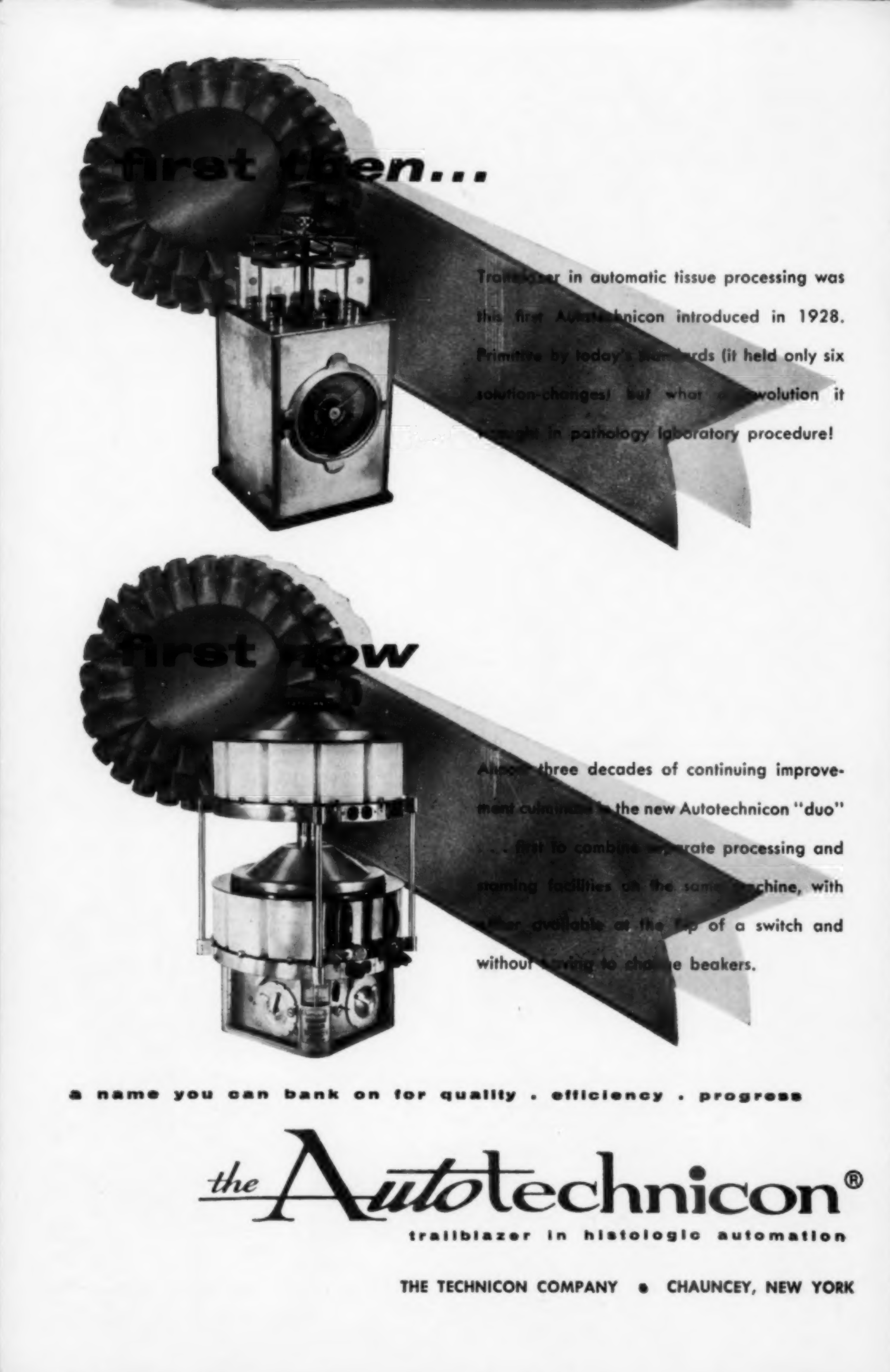
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